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The role of high affinity nicotinic acetylcholine receptors on anxiety-like behavior:  
a study in female mice

A thesis submitted in partial fulfillment of the requirements for the degree of Master  
of Science at Virginia Commonwealth University

By

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### List of Abbreviations

5-HT	5-hydroxy-tryptamine; serotonin
5-HT1A	5-hydroxy-tryptamine 1A; serotonin 1A
$\alpha$ 4HET	Alpha 4 Heterozygous
$\alpha$ 4KO	Alpha 4 Knockout
$\alpha$ 6L9'S	Alpha 6 Leucine 9' Serine
ACh	Acetylcholine
$\beta$ 2HET	Beta 2 Heterozygous
$\beta$ 2KO	Beta 2 Knockout
BAC	Bacterial artificial chromosome
BDZs	Benzodiazepines
CER	Conditioned emotional response
CNS	Central nervous system
CPP	Conditioned placed preference
CRH	Corticoid releasing hormone
CS	Conditioned stimulus
DA	Dopamine
DH $\beta$ E	Di-hydrobeta-erythroidine
EC50	Half maximal effective concentration
EPM	Elevated plus - maze
ES	Embryonic stem
EPSC	Excitatory postsynaptic current

EZM	Elevated zero - maze
GABA	$\gamma$ -aminobutyric acid
GABAA	$\gamma$ -aminobutyric acid A
GAD	Generalized anxiety disorder
HPA	Hypothalamic-pituitary-adrenal axis
IP	Intraperitoneal
IPSC	Inhibitory postsynaptic current
Kir2.1	Potassium channel subunit
LC	Locus coeruleus
LD	Light-dark
MAOIs	Monoamine oxidase inhibitors
MB	Marble-burying test
mPFC	Medial pre-frontal cortex
Nac	Nucleus accumbens
nAChR	Nicotinic acetylcholine receptor
NCS	The National Comorbidity Survey
NE	Norepinephrine
NPY	Neuropeptide Y
OCD	Obsessive-compulsive disorder
OF	Open-field
PTSD	Post-traumatic stress disorder
SAL	Saline
SN	Substantia nigra
US	Unconditional stimulus

VTA	Ventral tegmental area
WT	Wild type

## **Abstract**

### THE ROLE OF BETA2 SUBUNIT CONTAINING NICOTINIC ACETYLCHOLINE RECEPTORS ON ANXIETY-LIKE BEHAVIOR: A STUDY IN FEMALE MICE

By Jessicka Hall, B.Sc.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science, at Virginia Commonwealth University.

Virginia Commonwealth University, 2012.

Advisor: Darlene H. Brunzell, Ph.D., Dept of Pharmacology and Toxicology

Tobacco dependence is high in women who suffer from anxiety disorders yet little is known about the contributions of nicotinic acetylcholine receptors (nAChRs) on anxiety-like behavior.  $\beta 2^*$ nAChRs (\*denotes assembly with other subunits) are the most abundantly expressed nAChRs in the brain yet little is known about the contributions of  $\beta 2^*$ nAChRs on anxiety-like behavior in female mice. In this study, antagonism and nicotine effects on anxiety-like behavior was investigated across the life span in 6, 12 and 24-month-old drug-naïve knockout (KO), heterozygous (HET) and a gain of function  $\alpha 6L9S$  mice and wild type (WT). HET mice showed increased sensitivity to di-hydro-beta-erythroidine compared to WT mice. Aged mice showed decreased locomotor activity and exploratory behavior compared to younger mice. Low doses of nicotine produced anxiolytic-like effects, whilst a high dose of nicotine produced anxiogenic-like effects. Activation of the  $\alpha 6^*$ nAChRs supports an anxiolysis-like phenotype. These results implicate  $\alpha 4\beta 2^*$ nAChRs and  $\alpha 6\beta 2^*$ nAChRs in anxiety-like behavior.

## **Introduction**

Anxiety disorders are a major public health concern. In the United States, alone, tens of millions of people are affected by a range of clinically diagnosed anxiety disorders. It is estimated that 25% of all American adults have suffered from mental illness and nearly 50% of Americans will develop at least one mental illness during their lifetime (CDC, 2011a). Anxiety disorders are expensive to treat. The Agency for Healthcare Research and Quality's (AHR's) has reported that the annual costs associated with mental health in 2006 in the United States were over \$57 billion. Despite the healthcare dollars spent on this disorder, a majority of individuals with anxiety disorder are unresponsive to traditional treatment (Koen & Stein, 2011), indicating a need for novel therapeutics. This thesis will focus on nicotinic acetylcholine receptor subunit contributions to anxiety-like behavior in rodents. Understanding the molecular underpinnings of anxiety-like behavior may lead to more successful treatments for anxiety disorders and related health concerns.

### Anxiety and Tobacco Use

There are approximately 6 million annual tobacco-related deaths worldwide, all of which are preventable (World Health Organization, (MMWR, 2008). For many, anxiety disorders are also associated with nicotine dependence. Of the 6 billion smokers worldwide, subsets of these smokers suffer from psychiatric disorders (J. R. Hughes, Hatsukami, Mitchell, & Dahlgren, 1986; Kalman, Morissette, & George, 2005). Research has shown that people who suffer from anxiety disorders are twice



likely to smoke (Lasser et al., 2000), suggesting that individuals with anxiety may be more vulnerable to tobacco dependence. The National Comorbidity Survey (NCS) has reported a greater prevalence of nicotine dependence among patients who suffer from any anxiety disorders than among the general population (SAMHSA, 2004). Patients who suffer from anxiety and depression disorders have reported that they smoke to improve their symptoms of anxiety, mood and depression or to self-medicate (Kassel, Stroud, & Paronis, 2003; Khantzian, 1997). Anxiety is also an important factor in the psychopathology of schizophrenia where individuals have a four-fold elevated risk for smoking (Court et al., 2000; J. R. Hughes, Hatsukami, Mitchell, & Dahlgren, 1986). In addition to using tobacco to self-medicate symptoms of their disorders, those who suffer from mental illness may also have a shared vulnerability for their psychiatric and tobacco dependence (Khantzian, 1997; Morisano, Bacher, Audrain-McGovern, & George, 2009).

Research has shown that there is an increase in nicotine dependence and substance abuse amongst sufferers of psychiatric disorders (Kalman, Morissette, & George, 2005; Markou, Kosten, & Koob, 1998). Not surprisingly, studies have demonstrated that patients who suffer from anxiety and depression disorders have reported that they smoke to self-medicate and to improve their symptoms of anxiety, mood and depression (Kassel, Stroud, & Paronis, 2003; Khantzian, 1997). Nicotine is the major psychoactive ingredient in tobacco and is an agonist at nicotinic acetylcholine receptors (nAChRs) in the CNS and periphery (J. P. Changeux, Devillers-Thiery, & Chemouilli, 1984; J. P. Changeux et al., 1998; J. P. Changeux, 2010; M. R. Picciotto, 1998). Cigarette smoking is the leading preventable cause of death in developed countries and a major health concern in those with anxiety disorder who have twice the risk of tobacco dependence (Kalman, Morissette, &

George, 2005). The mechanisms involved in the anxiolytic effects of nicotine are not well understood. Therefore, using genetic and pharmacological tools, the aim of this thesis work is to elucidate the molecular mechanisms by which nicotine carries out its anxiolytic effect in mice.

### Nicotinic Acetylcholine Receptors and Anxiety

This thesis will investigate the contributions of high affinity nAChRs to affective behaviors in female mice. Nicotine is a major psychoactive ingredient in tobacco that acts on the brain centers associated with anxiety disorders by binding to nAChRs. nAChRs are ion channels located on the plasma membrane of neurons; when activated by nicotine or the endogenous ligand acetylcholine (ACh), nAChRs become permeable to cations that excite the cell. Nicotine competes with ACh for activation of the receptor. Animal and human studies have shown that nicotine's ability to reduce symptoms of anxiety is dose dependent (Juliano, Fucito, & Harrell, 2011; K. A. Perkins et al., 2006; M. R. Picciotto, Brunzell, & Caldarone, 2002). Nicotine binds to various central nervous system (CNS) nAChR subtypes and the subsequent release of neurotransmitters associated with emotional states relating to anxiety disorders is critical for the modulation of the symptoms of anxiety. This thesis will use mouse models to test the hypothesis that nicotinic receptors that contain a  $\beta 2$  subunit ( $\beta 2^*nAChRs$ ; \*denotes assembly with other subunits) regulate anxiety-like behavior in females.

## Neuronal Nicotinic Acetylcholine Receptors

nAChRs is a cholinergic receptor that belongs to the cys-loop receptor superfamily of ligand-gated ion channels. Cys-loop receptors have a canonical 13 amino-acid motif commonly flanked by a disulphide bridge. The receptor channel complex has a pentameric structure made up of 5 nicotinic subunits that form a central pore or channel through which cations flow. Each subunit is composed of 4 different membrane-spanning regions. The nAChR protein was initially identified as a protein from fish electric organ as a heteropentamer of about 300 000 MW with a ( $\alpha 1_2$ ,  $\beta 1$ ,  $\gamma 1$ ,  $\delta 1$ ) stoichiometry (J. P. Changeux et al., 1998). Further investigation has shown that this single molecular species carries two ACh binding sites located at interfaces between subunits; it has an ion channel along its transmembrane axis of pseudo-symmetry, and all the structural elements that mediate their coupling in the course of the activation and desensitization processes. Unlike peripheral nAChRs, which are found predominantly at the postsynaptic terminals of the neuromuscular junction, nAChRs in brain predominate on the soma and terminals of neurons where they modulate neurotransmitter release (for review see (J. P. Changeux et al., 1998). Currently, the sequences of 12 neuronal nAChR subunits have been established in brain (Corringer, Le Novère, & Changeux, 2000; McGehee & Role, 1995) nine are designated as  $\alpha$ -subunits ( $\alpha 2$ – $\alpha 10$ ) and share with electric organ  $\alpha 1$  subunit a pair of adjacent cystisines, while the others are referred to as non- $\alpha$  or  $\beta$ -subunits ( $\beta 2$ – $\beta 4$ ). All nAChR subunits share a similar hydropathy profile, consisting of a large globular extracellular N-terminal hydrophilic domain that carries the multiple loops and the neurotransmitter binding site, a trans-membrane domain made of four  $\alpha$ -helices (M1 to M4) about 20 amino acids long that defines the ion channel, and a cytoplasmic

domain. The cytoplasmic domain is inserted between the M3 and M4 and is believed to contribute to the anchoring of the receptor to the plasma membrane and to the modulation of the channels activity (J. P. Changeux et al., 1998).

The nAChRs are pentameric oligomers that undergo transitions for activation and desensitization. Investigation of the sequence of the known nAChR subunits indicates that these subunit genes share a common origin and have a long phylogenetic history. However, noticeable differences exist in the structure and variety of assembly of their subunits, their physiological and pharmacological properties, and their distribution in the brain (J. P. Changeux, Devillers-Thiery, & Chemouilli, 1984; J. P. Changeux et al., 1998; J. P. Changeux, 2010). Various combinations of both  $\alpha$ -type and  $\beta$ -type subunits form a wide variety of functional heterooligomers, with two ( $\alpha 2\beta 2$ ,  $\alpha 3\beta 2$ ,  $\alpha 4\beta 2$ ,  $\alpha 3\beta 4$ ,  $\alpha 6\beta 2$  etc.) or more ( $\alpha 3\beta 4\alpha 5$ ) ( $\alpha 1\beta 2\alpha 5$ ), ( $\alpha 3\beta 2\beta 4\alpha 5$ ) different subunits. These various combinations of nAChR subunits produce nAChRs with distinct pharmacological and physiological properties.

The nAChRs containing the  $\alpha$  and  $\beta$  subunits are the most abundantly expressed nAChRs in the central nervous system. Immunoprecipitation of rat brain tissue suggest that the  $\alpha 4$  and  $\beta 2$  subunits co-assemble with each other and not with the  $\alpha 7$  subunit (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; Sargent, 1993; Zwart & Vijverberg, 1998). Immunoprecipitation studies of have shown that nAChRs containing  $\alpha 4$  and  $\beta 2$  subunits account for a majority of the high-affinity  $^3\text{H}$ -nicotine and  $^3\text{H}$ -cytisine (for the  $\alpha 4$  nAChR) binding sites in rat brain (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; Sargent, 1993; Zwart & Vijverberg, 1998). Studies with  $\alpha$ -bungarotoxin a neurotoxic protein that binds competitively and irreversibly to

$\alpha 7$  but not  $\alpha 4$  and  $\beta 2$  nAChRs shows that the  $\alpha 7$  is the next largely distributed nAChRs to those that contain the  $\alpha 4$  and  $\beta 2$  (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; Sargent, 1993; Zwart & Vijverberg, 1998). The  $\alpha 7$  nAChR has a lower affinity for agonists such as nicotine and ACh (for review see (McGehee & Role, 1995)). The affinity binding of agonists such as nicotine and cytosine as well as  $\alpha$ -bungarotoxin binding are similar to those seen in human post mortem brain (Breese, Adams et al., 1997; Breese, Marks et al., 1997).

Nicotine can exert its effects by activation or desensitization of the nAChR. Activation of the nAChR leads to changes in behavior, similarly desensitization of the receptor can lead to a disruption of the transmission of endogenous ACh and the subsequent change in neuronal function that also leads to behavioral changes (for review see (M. R. Picciotto, Addy, Mineur, & Brunzell, 2008)). It is therefore important to investigate the behavioral effects mediated by the activation or desensitization of high the affinity nAChRs by nicotine.

It is not clear if nicotine regulates its effects on anxiety via activation or desensitization of the nAChRs. Animal research has shown that nicotine's activity at nAChRs is dose dependent and rewarding doses of nicotine activate nAChRs followed by rapid desensitization (J. P. Changeux et al., 1998; M. R. Picciotto, Brunzell, & Caldarone, 2002; M. R. Picciotto, Addy, Mineur, & Brunzell, 2008). Previous work has shown that sub-activating concentrations of nicotine reduces GABA release following nicotine pre-treatment (Lu, Marks, & Collins, 1999). Also, research has shown that chronic nicotine exposure results in an up-regulation of nAChRs (Kobiella et al., 2011; Lopez-Hernandez et al., 2004; Lukas, 1991; M. R. Picciotto, Brunzell, & Caldarone, 2002; M. R. Picciotto, Addy, Mineur, & Brunzell,

2008) and following chronic administration of nicotine a loss of nicotinic receptor functional activity occurs as a result of rapid and persistent desensitization (Eilers, Schaeffer, Bickler, & Forsayeth, 1997; Fenster, Rains, Noerager, Quick, & Lester, 1997; Hsu, Amin, Weiss, & Wecker, 1996; Katz & Thesleff, 1957; J. Lasalde-Dominicci et al., 2004; M. J. Marks, Farnham, Grady, & Collins, 1993; Peng, Gerzanich, Anand, Whiting, & Lindstrom, 1994; Sharp & Beyer, 1986; Vibat, Lasalde, Mcnamee, & Ochoa, 1995). This desensitized state has a higher affinity for acetylcholine and this state favors the anxiolytic-like effects of nicotine, research has shown that nAChRs desensitize with prolonged exposure to agonists with varying kinetics (J. P. Changeux, Devillers-Thiery, & Chemouilli, 1984; D. K. Williams, Wang, & Papke, 2011). Desensitization depends on concentration and exposure of agonists where long exposure leads to prolonged states that recover more slowly (Girod and Role, 2001). Similarly in human imaging studies, smoking of 2.4 cigarettes to satiate resulted in the occupancy of 80% receptors for up to 5 hours (Esterlis et al., 2010). This thesis will investigate the role the high affinity nAChRs play in nicotine-mediated anxiety behavior.

### High Affinity Nicotinic Receptors

The  $\beta 2^*$ nAChRs, (i.e.  $\alpha 4\beta 2$ ,  $\alpha 6\alpha 4\beta 3$ ,  $\alpha 4\alpha 5\beta 2$ ) make up a subclass of nAChRs to which nicotine binds with high affinity (Flores, Rogers, Pabreza, Wolfe, & Kellar, 1992; Sargent, 1993; Zhou et al., 2003; Zwart & Vijverberg, 1998)  $\beta 2^*$ nAChR has been found to be important for nicotine reward (Brunzell et al., 2006; Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Brunzell, 2012; Corrigan, Franklin,

Coen, & Clarke, 1992; De Biasi & Dani, 2011; Gotti et al., 2010; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; M. R. Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004).  $\beta 2$  knockout mice do not express conditioned place preference to nicotine and  $\beta 2$  subunit knockout mice self-administer cocaine but fail to maintain self-administration when the cocaine is switched to nicotine (Jackson, Walters, & Damaj, 2009; M. R. Picciotto et al., 1998). This suggests that  **$\beta 2^*nAChRs$**  are necessary for the maintenance of nicotine self-administration (M. R. Picciotto et al., 1998). Additionally, animals will self-administer a selective agonist of  $\beta 2^*nAChRs$ , suggesting that activation of these receptors is sufficient for nicotine self-administration (Liu et al., 2003). Other studies suggest that the  $\alpha 4$  and  $\alpha 6$  subunits that partner with  $\beta 2$  are also critical for nicotine conditioned place preference and nicotine self-administration (Avale et al., 2008; Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Brunzell, 2012; Gotti et al., 2010; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011; Orejarena et al., 2012), (Brunzell et al., 2006; Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Brunzell, 2012; Corrigall, Franklin, Coen, & Clarke, 1992; De Biasi & Dani, 2011; Exley et al., 2011; Gotti et al., 2010; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; M. R. Picciotto et al., 1998; Pons et al., 2008; Tapper et al., 2004). Compared to wild type mice,  $\alpha 4$  and  $\beta 2$  KO mice do not show an increase in striatal DA levels in response to nicotine, supporting the idea that  $\alpha 4\beta 2^* nAChRs$  are necessary for dopamine release (L. M. Marubio et al., 2003; M. R. Picciotto et al., 1998), a nicotinic response that is believed to be involved in dependence.

The  $\beta 2^*$ nAChRs have also been found to play a role in the anxiolytic effect of nicotine (Besson, Suarez, Cormier, Changeux, & Granon, 2008; King, Caldarone, & Picciotto, 2002; Labarca et al., 2001; McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011). The use of transgenic mice for the characterization of each subunit's role in nicotine's mediated response is extensive. Research has shown that homozygous mutant mice for the  **$\alpha 4$  subunit** showed increased basal levels of anxiety, which was attenuated by nicotine in the plus maze (Ross et al., 2000). In this study cDNA probe encoding the putative second transmembrane domain of the  $\alpha 4$  nAChR receptor subunit was cloned by PCR amplification of embryonic stem (ES) cell-derived genomic DNA using primers based on conserved regions between the human and rat published cDNA sequence. Studies by McGranhan and colleagues (2011) show that a low dose of nicotine elicited anxiolytic response in the elevated plus maze as shown by increases in the time spent in the open arms. This effect was not observed in  $\alpha 4$  nAChR subunit knockout mice. Labarca *et al.*, 2001 also show that mice heterozygous for a gain of function single point mutation in their  $\alpha 4$  nAChR subunit had increased anxiety behavior compared to wild type controls as measured by a decrease in open arm activity on the elevated plus maze. This highlights the importance of the  $\alpha 4^*$ nAChR receptor subunit in anxiety behavior.

Little is known about the role that  $\alpha 6^*$ nAChRs play in anxiety behavior. The  **$\alpha 6$  subunit** is a ligand binding subunit that co-assembles with the  $\alpha 4$ -,  $\beta 2$ - and  $\beta 3$ -subunits. The  $\alpha 6^*$ nAChRs are highly selectively expressed in DA neurons and is also found in the locus coeruleus and retinal ganglion cells and have the highest sensitivity to nicotine and ACh as shown by a 100-fold increase in  $EC_{50}$  value of  $\alpha 6^*$ nAChRs versus control (Salminen et al., 2007). These receptor subtypes are key



players in the cholinergic control of DA release. In contrast to knockout mutant mice, mutant strains have been generated that have a gain of function at the  $\alpha 6$  subunit. A mouse line the  $\alpha 6L9S$  has been generated in which a bacterial artificial chromosome (BAC) transgene was transfected into the mouse germline with a mutant copy of the mouse *Chrna6* gene that rendered mutant  $\alpha 6^*$  channels “hypersensitive” to endogenous ACh or exogenous nicotine. This resulted in the augmentation of DA neuron excitability and DA release in response to nicotine in these mice (Drenan et al., 2008). In this thesis  $\alpha 6L9S$  mice will be used to assess the role the  $\alpha 6$  subunit plays in anxiety behavior.

Chronic exposure to oral nicotine in female C57Bl/6 mice resulted in tolerance to the locomotor depressant and hypothermic actions of acute nicotine challenge. This tolerance was associated by an increase in  $\beta 2^*$ nAChR (i.e.  $\alpha 4\beta 2$ ,  $\alpha 6\alpha 4\beta 3$ ,  $\alpha 4\alpha 5\beta 2$ ) and  $\alpha 7$ nAChR number assessed by autoradiography techniques using  $^3H$ -cytisine and  $^{125}I$ -  $\alpha$ -bungarotoxin as radioligands (Sparks & Pauly, 1999). In contrast, a preponderance of the evidence shows that  $\alpha 6^*$ nAChR binding is decreased by chronic nicotine exposure (Champtiaux et al., 2002). Taken together, these results indicate that following chronic administration of nicotine there is change in the balance of high affinity nAChRs that may contribute to nicotine dependence and alter the effects mediated by nicotine.

It is important to note that nAChRs do not act alone and their function in the broader genetic context of multiple genes and biological cascades must also be considered. Some caution is needed for the interpretation of the results of studies using transgenic mice. nAChRs are expressed during embryogenesis and there is a

possibility that developmental compensation may occur either in the expression of other nAChR subunits or in related neurotransmitters. It is also important to note that there are behavioral differences that exist between mouse strains and when transgenic mice are used to investigate receptor function, mutations, unlike receptor agonists and antagonists, cannot be easily overcome in an *in vivo* preparation to provide a direct before-and-after comparison within the same organism. Nevertheless, transgenic animals allow us to observe the function of specific genes in the complex environment of a living organism, and transgenic techniques usefully complement traditional approaches.

#### Sex differences in high affinity nAChR expression

Sex differences in the availability of high affinity nAChRs containing the  $\beta 2$  subunit have been shown in smokers following abstinence. Using  $^{123}\text{I}$ -5-iodo-A-85380 single photon emission computed tomography to measure the availability of  $\beta 2^*\text{nAChRs}$  showed that male smokers showed greater availability compared to male non-smokers. This effect, however was absent in female smokers, female smokers did not show any difference compared non-smoking females.

Animal research has shown that chronic nicotine exposure in females has been found to result in increased anxiety-like behavior. However, in males, chronic nicotine administration results in decreased anxiety-like behavior (Caldarone, King, & Picciotto, 2008). It is unknown whether this may be due in part to changes in expression of  $\beta 2^*\text{nAChRs}$ . Our lab has shown that antagonism of the  $\beta 2^*\text{nAChRs}$  (i.e.  $\alpha 4\beta 2$ ,  $\alpha 6\alpha 4\beta 3$ ,  $\alpha 4\alpha 5\beta 2$ ) with the selective antagonist of  $\beta 2^*\text{nAChRs}$ , DH $\beta$ E, supports anxiolytic-like behavior in male C57BL/6 mice (unpublished findings). This

thesis work will use combine pharmacological and genetic approaches to explore how inactivation of the  $\beta 2^*nAChRs$  affects anxiety-like behavior in female mice.

### Sex as a determinant for smoking behavior

There are great costs associated with cigarette smoking. Cigarette smoking accounts for 1 in 5 deaths in the United States each year. Cigarette use is more common in men (21.5%) than women (17.3 %) (CDC, 2011b), but tobacco related deaths in women have increased steadily over the past 10 years (K. A. Perkins, Donny, & Caggiula, 1999; Wetter et al., 1999). Women who smoke are more likely than men to report that smoking cigarettes relieves anxiety (Marqueta, Nerin, Jimenez-Muro, Gargallo, & Beamonte, 2012; K. A. Perkins, Donny, & Caggiula, 1999; Wetter et al., 1999). Women are more likely to report anxiety as a reason for relapse to smoking (Xu et al., 2008). Although globally there is a higher rate of smoking for men (40%) than women (approximately 9%) as of 2006 (World Health Organization), anxiety disorders have a higher prevalence (23.8%) among females than male (15.6%) smokers (Kessler, 2005). There is also a growing incidence of anxiety disorders among children and young adults (Kessler, 2005, SAMHSA 2010). Initiation of smoking is on the rise in young girls (CDC, 2010; Nichter, Nichter, Vuckovic, Quintero, & Ritenbaugh, 1997) but it is not clear if anxiety is a major factor contributing to smoking during adolescence.

Tobacco related mortalities have increased equally amongst men and women and although men tend to smoke more than women, women have been found to have greater physical and emotional dependence on smoking as measured by increased relapse rate due to pre- and post-cessation stressors (Bjornson et al.,

1995; K. A. Perkins, Donny, & Caggiula, 1999; Xu et al., 2008). Research has shown that female smokers also have higher relapse rate and fewer attempts to quit smoking compared to male smokers (Bjornson et al., 1995; Wetter et al., 1999). Similarly to human studies, in rodent models females also show greater vulnerability to nicotine use compared to males has shown by acquisition of self-administration of lower doses of nicotine, a reduction in latencies to earn their first infusions of nicotine and an enhancement of the rewarding effects in nicotine placed preference in adult females compared to male (E. C. Donny et al., 2000; Torres, Natividad, Tejeda, Van Weelden, & O'Dell, 2009). Not surprisingly, women are more likely to suffer from depression and anxiety disorders (Castle, Kulkarni, & Abel, 2006; Gater et al., 1998) and women have also greater tendency than men to smoke in order to relieve anxiety as measured by higher relapse rate and smoking more cigarettes (Marqueta, Nerin, Jimenez-Muro, Gargallo, & Beamonte, 2012; Wetter et al., 1999). The mechanism of how smoking may relieve anxiety is largely unknown. Using a combination of genetic and pharmacological manipulations these studies will assess the contributions of  $\beta 2^*nAChRs$ ,  $\alpha 4\beta 2^*nAChRs$  and  $\alpha 6\beta 2^*nAChRs$  to anxiety-like behavior in mice.

### Anxiety in Humans

Anxiety can be defined as a subjective emotional response to a stressor that involves a “tense, unsettling anticipation of a threat to a vague event” (Rachman, 2004). Anxiety is a “negative affect related to fear that is diffuse, objectless, unpleasant and persistent” (Rachman, 2004) produced by cognitive processes mediating the anticipation, interpretation or recollection of perceived stressors and

threats (Charney DS and Drevets WC, 2002). During an anxious episode the sufferer often has difficulty in the identification of the cause of the uneasy tension or the nature of the anticipated threat. These emotional-behavioral responses can be brought about by response to exteroceptive visual, auditory, olfactory or somatosensory stimuli or to interoceptive input through the viscera and the endocrine and autonomic nervous systems (Charney DS and Drevets WC, 2002).

This definition implies that anxiety is a subjective experience, involving more than just physical stressors. Anxiety is commonly evaluated clinically in humans by verbal reports. However, anxiety in humans is also associated with nonverbal changes in behavior such as increased heart rate, blood pressure and muscle tension and palmar sweating (Caruso, 1990). The use of verbal reports is not possible for the assessment of experimental or clinical anxiety-like behavior in animals. Research has shown that rodents share the same hormones associated with stress in humans. Mice lacking the corticotrophin-releasing hormone gene exhibit reduced anxiety-like behavior compared to control (Timpl et al., 1998). In order to evaluate anxiety pre-clinically in rodents, investigators rely on the evaluation of behaviors that correlate with physiological stress responses seen in both animals in humans as well as behaviors that are responsive to anxiolytic drugs.

Emotional expression involves a range of behavioral, endocrine and autonomic manifestations of the emotional response (Charney & Drevets, 2002; Fellous, 1999) that is often relayed as a subjective feeling that accompanies the response. Emotional processes are modulated by complex neurobiological systems that modulate behavior. Anxiety disorders develop when the aspects of emotional processing modulated by complex neurobiological systems are maladaptive and symptoms become persistent (lasting at least 6 months).

Anxiety disorders commonly occur along with other mental and physical disorders that may mask or make anxiety symptoms worse. Anxiety disorders encompass different disorders including panic disorder, post-traumatic stress disorder (PTSD), obsessive-compulsive disorder (OCD), social phobia and generalized anxiety disorder (GAD). Each anxiety disorder listed above has different symptoms, but all the symptoms involve excessive, often irrational fear and dread.

**Panic disorder** is characterized by sudden attacks of terror, usually accompanied by changes in the autonomic nervous system such as pounding heart, sweatiness and weakness. Panic attacks are usually associated with a fear of impending doom or a fear of losing control. Panic disorder affects approximately 6 million American adults (Kessler RC, Chiu WT, Demler O, Walters EE., 2005) and is twice as common in women as men (Kessler RC, Berglund PA, Demler O, Jin R, Walters EE., 2005). Panic attacks often begin in late adolescence or early adulthood (Kessler RC, Berglund PA, Demler O, Jin R, Walters EE., 2005).

**PTSD** typically develops after a terrifying incident that involved physical harm or the threat of physical harm. Sufferers of PTSD repeatedly relive the trauma in their thoughts during the day and in nightmares when they sleep. This can result in the patient losing touch with reality and believing that the traumatic incident is happening all over again. PTSD affects roughly 7.7 million Americans and women are more likely to develop PTSD than men; PTSD can develop at any age but is most common in adulthood (Kessler RC, Berglund PA, Demler O, Jin R, Walters EE., 2005).

People with **OCD** have persistent, obsessions and use rituals (e.g. excessive washing or checking) to control the anxiety these thoughts produce. These rituals may result in debilitating consequences that affect the patient's quality of life. OCD

affects about 2.2 million American adults and it affects men and women equally; OCD usually appears in childhood, adolescence, or early adulthood.

**Social phobia** occurs when a person becomes overwhelmingly anxious and excessively self-conscious in normal social situations. Social phobia affects about 15 million American adults and is more prevalent in women than men; this disorder usually develops in childhood or early adolescence.

People with **GAD** are characterized as having hyperbolic tension and worry. GAD affects approximately about 6.8 million American adults and is twice more prevalent in women than men (Robins & Regier, 1991); GAD develops gradually and take effect at any time, however, the highest risk occur between childhood and middle age. Anxiety disorders affect a large number of the American population, it is therefore important to elucidate the mechanisms involved in anxiety disorders to better develop effective therapeutics for these often time debilitating disorders.

**Clinical human studies** of anxiety disorders include randomized placebo-controlled double-blind trials of novel pharmacotherapies, imaging methodologies and gene expression with pharmacotherapy studies in order to better develop new treatment targets, and in order to help personalize future treatment strategies. The benefit of human studies of anxiety disorder is that there is better assessment of the subjective effects and investigators can better assess the effectiveness of treatment. One major setback to studies in humans is that the disorder cannot be easily studied mechanistically. Imaging studies in and of themselves can create anxiety confounding experimental outcomes and human tissues is often compromised in post-mortem studies and are confounded by therapeutic history in treatment of anxiety disorders.

## Sex Hormones and Anxiety

Women of reproductive age are more prone to developing major depressive and anxiety disorders than men, the prevalence being at least two times higher in women (Kessler, McGonagle, Swartz, Blazer, & Nelson, 1993; Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Weissman & Olfson, 1995). Gender differences typically emerge during puberty (Bouma, Ormel, Verhulst, & Oldehinkel, 2008) and afterwards the prevalence rate in women remains higher until menopause (Kessler, 2003). The different phases in the reproductive life of women and/or female hormonal fluctuations appear to play a role in the gender differences observed. Studies have attributed the gender differences in lifetime prevalence rate of affective disorders such as anxiety to the cyclic release of female sex hormones particularly estrogen (Desai & Jann, 2000; Steiner, Dunn, & Born, 2003). Typically models for anxiety-like behavior are conducted in males. A major reason for using male mice is that the cyclic release of female sex hormones, estrogen and progesterone, in female mice produces a further and unstable endogenous factor in the models that by itself can have significant outcome on physiology and behavior of the animal. Since these studies will be performed in females whose gonads are intact, this may introduce variability in our dataset that will require increasing our n sizes. Although it is important to assess the contributions of sex hormones on anxiety, studies have shown that vaginal mechanical stimulation of the internal and external genital structures in spontaneous ovulators (e.g. rats, humans, mice, and cattle) induces estrus and prolongs and intensifies the immobile mating stance (lordosis) in female rats. Studies have shown that common methods of taking vaginal smears in female rodents often lead to estrus in animals in which true estrus cannot occur and the procedure itself disturbs the regularity of the cycle (Emery & Bchwabe, 1936; Emery



& Schwabe, 1938; P. V. Rogers & Allen, 1937; Wade & Doisy, 1935). Extraction of serum from the blood is another method for assessing sex hormone levels in rodents but previous studies have shown that it is extremely difficult to accurately measure hormone levels in mice (Frick, Fernandez, & Bulinski, 2002) due to the appreciable amount of blood needed for these measurements. The present studies are performed only in females. Detection of dichotomies between male and female anxiety-like behavior in future studies could be followed-up with experiments that use gonadectomy and hormone replacement to determine if sex hormones are driving the behaviors observed in rodent studies (Russo et al., 2003).

#### Anxiety-like Behavior in Rodents

Humans and mice share a close genetic and physiological relationship where the human and the mouse genome share 85% identity. These genomes share similar susceptibility to many simple and complex genetic diseases. Intensive inbreeding has provided many genetically isogenic strains with phenotypically distinct features that have been successfully used to identify and often define the genetics of many models of human disease. Different mouse strains provide a resource for examining the genetic diseases including those that include drugs of abuse. However, caution is needed when embarking upon experiments using the mouse model systems. An important factor to consider is that alteration or deletion of a gene leads to compensatory mechanisms that are present over the lifetime of the animal. This makes it difficult to determine whether the effects observed in genetically modified mice are due the alteration of a gene, or if they are due to compensatory changes that occur during development. Therefore, each experiment

and finding must be evaluated as to species-specific responses to stressors, suitability of experimental manipulations, equipment and assessment strategy and strain and species limitations in behavioral and adaptive repertoires.

**Preclinical studies** using animal models that model human pathological anxiety in rodents are a useful way of mechanistically identifying potential molecular targets for treatment of anxiety. Animal models shown to respond to drugs that are anxiolytic in humans are believed to have some predictive validity for therapeutic efficacy and may identify new therapeutic targets for treatment of anxiety. Non-human animals possess the same neurotransmitters and brain areas that regulate anxiety. Animal studies can control for behavioral and environmental factors that are difficult to control in humans. In these studies we will use mice. Mouse models benefit from transgenic approaches that can be used to study molecular contributions to anxiety-like behavior (for review on see (Cryan & Holmes, 2005).

Preclinical animal models use non-verbal behavioral tests for the assessment of anxiety-like behavior that include: exploration-based conflict tasks and non-exploration based tests. Most ethological procedures in anxiety models are designed to trigger relevant ethological conflict/conditioned behaviors. Exploration-based conflict tasks are the most commonly used anxiety-like tests in rodents. Rodents such as mice have an innate aversion to open and brightly lit spaces. Conversely, mice will naturally explore new environments. Exploration-based conflict tasks take advantage of the competing ethological drives of mice to avoid predation and to explore a novel environment. Exploration-based conflict tasks such as the **elevated plus maze**, the **mirror task**, the **open field**, and the **light-dark task** utilize test apparatuses where the mouse's drive to approach is in disagreement with the avoidance of the potential threat. These tests are sensitive to locomotor effects of

drugs, so that locomotor activity should be assessed for potential confounding effects in these tasks.

Conversely, non-exploration based tests, e.g. **conditioned inhibition**, **marble burying** and **acoustic startle response** do not rely on exploration and hence allow the measurement of anxiety-like behavior where it is less difficult to separate changes in locomotor activity from anxiety-induced changes in activity. As with the conflict tasks, drugs with anxiolytic action in humans produce decreases in avoidance of aversive or threatening areas in (non- mutant) mouse strains and anxiogenic drugs have been shown to have the opposite effect (K. R. Bailey & Crawley, 2009; Holmes, 2003).

Various methodical procedures are employed when designing experiments. These procedures include the environmental condition in which the animals are housed (this should be kept consistent across experiments), food and water access should be *ad libitum*, experimental history of subject, prior test and drug exposure and housing.

The aversive or threatening area in animal models of anxiety can take various forms; an open, elevated arm (**elevated plus maze**), open, elevated quadrant (**elevated zero maze**), light compartment or arena (**light- dark exploration test**, **emergence test**), mirrored arena (**mirrored chamber test**), a staircase (**staircase test**), an area in which a predator was previously encountered (**mouse defense test battery**, **predator exposure test**), or central area of a novel or brightly lit open fields (**open-field test**).

Studies have shown that drugs known to alleviate anxiety in humans produce reduced anxiety-like behavior in animal models (Calabrese, 2008; Turner, Castellano, & Blendy, 2010; Turner, Castellano, & Blendy, 2011). The **open-field**

(OF) test allows for the assessment of novel exploration and general locomotor activity. Two factors have been shown to influence anxiety-like behavior in the OF test, these are social isolation and stress created by brightly lit, uncovered, novel environment. Subjects are placed in an open area and dependent measures are analyzed such as time spent in the center of the arena. Experiments are usually conducted in brightly lit conditions.

Increased digging behavior in rodents is associated with physiological stress responses and is decreased by treatment with anxiolytics (Njunge & Handley, 1991). The **marble-burying test** is a paradigm used to assess drugs used to treat anxiety in humans. This ethological test assesses non-exploratory behavior and consists of a cage filled with loose bedding and identical glass marbles placed at equidistance from each other. Previous work has shown that benzodiazepines known to relieve anxiety in humans reduce the number of marbles buried in rodents (Nicolas, Kolb, & Prinssen, 2006; Njunge & Handley, 1991). A reduction in the number of marbles buried is interpreted as reduced anxiety-like behavior in rodents.

The **light-dark exploration test** (LD) assesses the ethological drive of rodents to avoid predation by hiding in an enclosed dark chamber and to explore their environment. The apparatus for the LD test typically consists of a small dark enclosed chamber and a larger, open, brightly lit chamber. Rodents find lighted open areas aversive and this inhibits the ethological drive in rodents to explore a novel environment. An increase in exploratory activity is interpreted as a release of exploratory inhibition and is thus interpreted as decreased anxiety-like behavior. Dependent variables include total locomotor activity counts in both chambers and time spent in the light chamber. When animals start in the dark chamber, latency to enter the light chamber can also provide a measure of anxiety-like behavior.

Typically an enhancement of exploratory behavior should not be due to general increases in locomotor activity (Bourin & Hascoet, 2003). Overall changes in locomotor activity can be assessed by comparing activity in the light and dark chambers.

Previous work has shown that the LD assay, MB test and OF tests are valuable tools in the assessment of the anxiety-phenotype (Bourin & Hascoet, 2003; J. Cao et al., 2010; Nicolas, Kolb, & Prinssen, 2006; Njunge & Handley, 1991).

The **elevated plus- maze or elevated zero- maze** (EPM/EZM) takes advantage of the natural tendency of mice to explore novel environments. The EPM was originally designed in 1985 as a screening tool for the assessment of anxiolytic and anxiogenic drugs on exploratory activity in rodents. As stated above this test takes advantage of the approach-avoidance conflict behavior that is associated with an increase in physiological stress indicators. The apparatus EPM consists of 2 opposite closed arms (alleys with walls) and 2 perpendicular and opposite open arms (alleys with no walls). Like the open field, EPM experiments are also conducted in brightly lit conditions. Animals are allowed to freely explore the different arms with the open arms being presumably more aversive since mice spend much less time there. Anxiolytic drugs help to reduce the inhibition of open arm exploration (Calabrese, 2008). Typical dependent variables include the number of open and closed arm entries and time spent in open and closed arms. An advantage of using this model is that, unlike LD and OF, behavior is consistent over multiple days and hence can be utilized over several days of training.

An advantage of using ethological tests such as EPM, LD, OF and MB is that no prior training is needed and animals do not need to be food or water deprived. Other animal models of anxiety that require prior training include the **fear conditioning**,

**conditioned emotional response (CER), fear potentiated acoustic startle**, and the **place conditioning** paradigms. These models must include behavioral controls to assess if changes in anxiety-like behavior are learning-dependent.

**Fear conditioning, potentiated acoustic startle and conditioned emotional response** involve a learned emotional anxiety or fear reaction to in response to predictive cues associated with an aversive stimulus. These Pavlovian measures involve the presentation of a conditioned stimulus (CS) (e.g. light and tone) with an unconditional stimulus (US) (e.g. foot shock). After several pairings, the US comes to elicit the unconditional response (UCR). In fear conditioning and CER the CS evokes freezing and a suppression of lever-pressing respectively. With CER, the disruptive effect of the CS on goal-oriented behavior is termed conditioned suppression. Conditioned suppression is the amount of suppression of on-going operant behavior and is used as an indicator of the strength of fear. In contrast, an aversive CS. CER enhances acoustic startle reflexes and acoustic startle responses are reversed by drugs that are used to treat anxiety disorders in humans such as benzodiazepines (chlordiazepoxide, diazepam and oxazepam) (for review see (Davis, 1990).

During **place conditioning** or **conditioned avoidance** a stimulus such as a drug or a foot shock is paired with an environment that the animal can voluntarily choose to spend time in during a test. Dependent measures are latency to enter or leave the conditioned chamber and time spent in the chamber where animals received a shock. Place conditioning is a useful behavioral model to study the rewarding and aversive effects of a drug. The apparatus commonly consisting of a three-compartment chamber designed to have different characteristics. The center compartment has no discernible characteristics and is not paired with a drug. The

gates between the compartments can be left opened to allow an animal to pass freely between them. During training an animal is given an injection with a drug and consistently placed in a paired compartment for several minutes. Control injections are followed by placement in the other compartment. Training typically involves 3-4 sessions for each drug and vehicle. On test day, the animal is allowed to move freely between compartments. Dependent measures include the time spent in the drug-paired compartment. Decreases and increases from baseline determine if the drug was respectively aversive or rewarding during training (Brunzell, Mineur, Neve, & Picciotto, 2009; Prus, James, & Rosecrans, 2009). This thesis work utilizes a number of behavioral tasks to assess anxiety-like behavior in mice; these include the OF, MB and the LD.

### Physiology and Anatomy of Anxiety

The physiology of emotional states relating to anxiety disorders is found in the lower regions of the brain. These regions include the limbic system, diencephalon, the reticular formation and its connecting brain circuitry. The neuroanatomical circuits associated with anxiety are modulated by a variety of chemical neurotransmitter systems. These include neurotransmitters whose release is modulated by nAChRs: norepinephrine (NE), serotonin (5-hydroxy-tryptamine, 5-HT), dopamine (DA), and  $\gamma$ -aminobutyric acid (GABA) (J. P. Changeux et al., 1998; M. R. Picciotto, Brunzell, & Caldarone, 2002). Nicotine also regulates peptidergic neurotransmitters and hormones, corticotropin releasing hormone (CRH), neuropeptide Y (NPY) substance P, adenosine, and cytokines, suggesting a role for nAChRs in their modulation (Millan, 2003). The best-studied neurotransmitter systems connected with anxiety

involve stress hormones of the hypothalamic-pituitary-adrenal (HPA) axis and the central noradrenergic system. These neurochemical systems are important for adaptive functions in preparing the organism for responding to the stressor by the modulation of various survival mechanisms. When dysfunction occurs, these biological responses to threat or stress may become maladaptive if they are chronically or inappropriately activated.

Disruption of the serotonergic, noradrenergic, dopaminergic and/or GABAergic pathways has been implicated in supporting anxiety disorders.

Ascending **serotonergic** projections originate from cell bodies located along the midline of the brain stem, in the raphe nuclei in pons and midbrain that project to forebrain. This pathway plays an important role in the regulation of responsiveness of cortical neurons involved in mood. Projections from the raphe nuclei project to brain regions such as the prefrontal cortex and forebrain. Studies have shown that elevated raphe nucleus 5-HT levels are associated with reduced anxiety in rats exposed to stress as shown by increased entries in the open arms of the elevated plus maze (Kaiyala, Vincow, Sexton, & Neumaier, 2003). Exposure to various stressors results in increased 5-HT metabolism in the medial pre-frontal cortex (mPFC), amygdala, lateral hypothalamus and nucleus accumbens in rat brains (Inoue, Tsuchiya, & Koyama, 1994).

Ascending **noradrenergic** projections originate from cell bodies located in the dorsal and ventral medulla. NE-containing neurons located in the locus coeruleus (LC) provide important projections to the cerebral cortex and cerebellum and are important for maintaining responsiveness to unexpected stressors. Projections from the LC that extend to the limbic system regulate anxiety (Cheeta, Irvine, Kenny, & File, 2001; J. M. Weiss et al., 1994). Drugs used to treat anxiety in humans such as



benzodiazepines have been found to inhibit LC activity in rodents (Kozak, Valzelli, & Garattini, 1984), which is thought to support the anxiolytic effects of these drugs. However, some studies have found that local LC infusion of drugs that decrease neuron firing resulted in anxiogenic behavioral responses (J. M. Weiss et al., 1994). Conversely drugs used to increase the firing of cells in the LC resulted in anxiolytic responses (J. M. Weiss et al., 1994). The mesolimbic system is one of the major DA pathways in the CNS. Ascending **dopaminergic** projections originate in the ventral tegmental area (VTA) and the substantia nigra (SN). Dopaminergic neurons in the ventral tegmentum (mesocortical pathway) project to the frontal lobes where they contribute to the emotional response to anxiety. Studies in which a mutant strain of mice for the *Clock* gene  $\Delta 19$ , which has a complete behavior profile similar to human mania when injected with an inwardly rectifying potassium channel subunit (Kir2.1) into the VTA, which mimics the effects of the treatment for mania with lithium, mutant mice showed anxiogenic behavior compared to control mutant mice virally transfected with green fluorescent protein (Coque et al., 2011). Nicotine mediates reward and aversive motivational effects in humans and animals. The VTA is believed to play a crucial role in mediating the rewarding and aversive properties of nicotine. Laviolette and van der Kooy (2003) have shown that blockade of mesolimbic DA transmission blocks nicotine aversion and increases the sensitivity to the rewarding effects of nicotine following local administration into the VTA (Laviolette & van der Kooy, 2003). This suggests that VTA DA signals may be anxiogenic and further highlights the importance of the mesocortical pathway in anxiety. The mesolimbic pathway extends from the VTA of the midbrain to areas of the limbic system. The limbic system includes the nucleus accumbens, amygdala and hippocampus and is associated with reward and pleasure as well as

motivational valence for aversive behaviors. The limbic system, mesocortical pathway and the nigrostriatal pathway are involved in the inhibitory function of dopaminergic neurons. Acute stress leads to an increase in DA release from nerve terminals and metabolism in many brain regions. Research has shown that the mesolimbic dopamine system plays an important role in anxiety behaviors (Barrot et al., 2001; Barrot et al., 2002; Barrot et al., 2005; Olson et al., 2005). GABA is the major inhibitory neurotransmitter in the CNS and is found in nearly every region of the brain. GABA receptor function can be altered by exposure to stress and activation of this pathway leads to decreased anxiety. Central GABA<sub>A</sub> receptors are expressed throughout the brain, but are most densely concentrated in the cortical gray matter. Central GABA<sub>A</sub> receptors agonists potentiate and prolong the synaptic actions of the inhibitory neurotransmitter, GABA, by increasing the frequency of GABA-mediated chloride channel openings (D. W. Choi, Farb, & Fischbach, 1981; Millan, 2003).

Taken together, these neural networks provide a various mechanisms by which a stressor can influence behavior and mood.

### Treatment for Anxiety Disorders

Typically, anxiety disorders are treated with medications, psychotherapy or a combination of the two. Psychotherapy such as cognitive-behavioral therapy is a useful tool for treating anxiety disorders (Otte, 2011). An **anxiogenic** agent is one that causes an increase in anxiety. In comparison **anxiolytic** agents are those that reduce or relieve anxiety. Typically, treatment with anxiolytics act by inhibiting neuronal activity in brain structures that mediate fear expression and the behavioral

sensitization and facilitation of endogenous mechanisms necessary for the modulation of the neural transmission of information about aversive stimuli and responses to such stimuli. Anxiety disorders are principally managed with medications (Koen & Stein, 2011) that include, selective 5-HT reuptake inhibitors (SSRIs) antidepressants such as fluoxetine (Prozac), serotonin (5-HT)<sub>1A</sub> receptor agonists, tricyclics such as imipramine (Tofranil), monoamine oxidase inhibitors (MAOIs) such as phenelzine (Nardil), beta-blockers such as propranolol (Inderal) and drugs that enhance GABA receptor activity such as benzodiazepines (BDZs) and buspirone.

Given self-reports suggesting that cigarettes and smokeless tobacco relieve anxiety; it is possible that nAChRs may promote anxiolysis. nAChRs are ubiquitously expressed throughout the brain including limbic structures such as the amygdala, prefrontal cortex, hippocampus and sensory thalamus that regulate anxiety (J. P. Changeux et al., 1998; M. R. Picciotto, Brunzell, & Caldarone, 2002). The nAChRs reside predominantly on neuronal soma and axon terminals where they modulate the release of GABA, DA, NE and 5-HT in response to stimulation by nicotine or the endogenous neurotransmitter ACh (J. P. Changeux et al., 1998).

### Age differences and Anxiety

Along with sex difference, the prevalence of anxiety is also age-dependent. Young adults with nicotine dependence had higher rates of major depression and anxiety disorders (Breslau, Kilbey, & Andreski, 1991). The properties of the nAChRs on dopamine terminals change with age (Alves, Bailey, Nashmi, & Lambe, 2010) and during early development there is a large distribution of  $\alpha 4\beta 2$ \*nAChRs in the cortex

and changes in the expression of  $\alpha 4\beta 2^*$ nAChRs differentially affects anxiety-like behavior in young and aged mice. Studies in mice report that aged (24 to 28-month-old) mice have reduced expression of  $\alpha 4\beta 2^*$ nAChRs compared to young (4 to 6-month-old) mice (S. W. Rogers, Gahring, Collins, & Marks, 1998). Similarly, human imaging studies the elderly have shown a decrease in nAChR availability as we age (Mitsis et al., 2007; Mitsis et al., 2009). In males, aged rats shown increased anxiety compared to younger rats (Meyza, Boguszewski, Nikolaev, & Zagrodzka, 2011; Pietrelli, Lopez-Costa, Goni, Brusco, & Basso, 2012). Taken together, these results show the importance of investigating the role nicotinic receptors play in age-dependent changes in anxiety. This thesis will compare anxiety-like behavior across the life span in 6, 12 and 24-month-old female wild type,  $\alpha 4$ nAChR subunit knockout ( $\alpha 4$ KO) mice and  $\alpha 4$ nAChR heterozygous ( $\alpha 4$ HET) mice.

### Experimental procedures

In these experiments to assess the contributions of the  $\beta 2^*$ nAChRs to anxiety-like behavior in female mice a number of mouse models of anxiety-like behavior was used. Behavior was initially assessed in the light-dark assay, followed by the marble burying test and the open field test of the locomotor activity assay.

To assess the contribution of  $\beta 2^*$ nAChRs to anxiety-like behavior in middle aged and aged females, 12- and 24-month-old mutant mice with a null mutation ( $\beta 2$ KO) of their  $\beta 2$  subunits and heterozygous ( $\beta 2$ HET) animals with a 50% reduction of their  $\beta 2^*$ nAChRs were compared to wild type controls (WT) in a light-dark assay, marble burying task and open field test. To pharmacologically assess if activation or inhibition of  $\beta 2^*$ nAChRs promotes anxiety-like behavior; mice were injected with 0 or

2.3 mg/kg i.p., DH $\beta$ E prior to testing in the light-dark and marble burying task. To further assess the stoichiometry of  $\beta$ 2\*nAChRs that contribute to anxiety-like behaviors across the lifespan, 6-, 12-, and 24-month old female mice with a null mutation of their  $\alpha$ 4 subunits ( $\alpha$ 4KO),  $\alpha$ 4 nAChR subunit heterozygous ( $\alpha$ 4HET) and wild type control mice (WT) were also studied in a light-dark assay and marble burying task following injection of saline vehicle or 2.3 mg/kg i.p. DH $\beta$ E. As with  $\beta$ 2\*nAChR mutant mouse studies,  $\alpha$ 4 mice were assessed in the open field in the absence of drug injection.  $\beta$ 2\*nAChRs also assemble with the  $\alpha$ 6 subunit both in the presence or absence of  $\alpha$ 4. To assess the role of the  $\alpha$ 6 $\beta$ 2\*nAChRs to anxiety-like behavior, adult, female mice with a single point mutation in their  $\alpha$ 6 subunit which renders them hypersensitive to nicotine ( $\alpha$ 6L9S) were compared to their wild type (WT) littermate controls in the light-dark assay and marble burying task. Mice were injected with 0, 0.001, 0.0032, 0.01, 0.032, 0.1 and 0.32 mg/kg i.p., (freebase) nicotine prior to anxiety assays as well as prior to a locomotor task.

## Materials and Methods

### Animals

Female wild type, knockout, or transgenic mice on a C57BL6J background were bred in a vivarium at VCU for all experiments and were housed in cages of groups of 3-6. Mice were separated into 3 groups by age with 6-month-old animals representing adult mice, 12-month-old mice considered middle aged and 24-month-old mice representing the aged group for these experiments.  $\beta 2$  ( $\beta 2$ KO) and  $\alpha 4$  ( $\alpha 4$ KO) subunit null mutant mice were generated from heterozygous matings backcrossed at least 14 generations onto a C57BL6J background. Heterozygous mice that have a 50% reduction in their respective  $\beta 2^*$  ( $\beta 2$ HET) and  $\alpha 4^*$ nAChRs ( $\alpha 4$ HET) were also studied and wild type mice served as controls.  $\alpha 6$  subunit bacterial artificial chromosome transgenic gain of function mice with one allele containing a single point mutation of serine to leucine in the pore-forming region of the M2 domain ( $\alpha 6$  L9S) were crossed to C57BL6 mice (Drenan et al., 2008). Ear punches were used to identify animals and confirm genotype using PCR analysis. Mice were housed 3-5 per cage and maintained in a temperature- and humidity - controlled vivarium (20-22 °C) under a 12: 12 h light-dark cycle (lights on at 06:00 hours). Food and water were available *ad libitum*. Mice were habituated to handling at least 3 days before behavioral testing. Behavioral tests were conducted between 0800 and 1700 hours. All studies were approved by the Virginia Commonwealth University Animal Care and Use Committee and followed National Institutes of Health Guide for the Care and Use of Laboratory Animals.

## Drug Dosing and Administration

Mice undergoing behavioral testing received intraperitoneal (i.p.) injections of di-hydrobeta-erythroidine (DH $\beta$ E) (Tocris Bioscience; Bristol, U.K.) or nicotine hydrogen tartrate (Sigma Aldrich; St. Louis, MO, U.S.A). DH $\beta$ E in 0.9% sterile saline was administered at 0 or 2.3 mg/kg (freebase) 15 minutes prior to behavioral testing and placed in a novel holding cage. The following nicotine doses in 0.9% sterile saline were administered to subjects immediately prior to testing: 0, 0.001, 0.0032, 0.01, 0.032, 0.1 and 0.32mg/kg (nicotine doses are expressed as freebase).

## Apparatus

### Light-dark Assay

Light-dark experiments were conducted in modified Med Associates place conditioning chambers (St. Albans, VT, U.S.A.). The dimensions of the chambers were: light (L 26.5 cm x W 12.7 cm x H 26.2 cm) and dark (L 16.8 cm x W 12.7 cm x H 12.7 cm), with the light chamber being open (no ceiling) and illuminated with a single 23W n: vision fluorescent LED light bulb. The adjacent dark chamber was enclosed with a dark lid and the experimental room was unlit except for the illumination provided by the light chamber. There was a (10.3 cm x 5.3 cm) door that enabled movement between chambers. Data was collected on a PC computer using Med Associates software.

### Marble Burying Test

Marble burying experiments were conducted in standard mouse polycarbonate cages (30 cm L x 18 cm W x 18.5 cm H) filled with 5 cm of loose sawdust bedding. The outer perimeter of the cages covered by white card-stock to occlude visualization of other mice. Cages were covered with a transparent Plexiglas lid with 3 (0.9 cm in diameter) holes drilled in the center. 20 glass marbles of the same size and color were placed atop the sawdust in a 4 x 5 grid in the cage with a 1-inch perimeter between the marbles and the walls of the polycarbonate cage.

### Open-field Test

The open-field experiments were conducted in a translucent, square experimental chamber (42 cm L X 42 cm W X 30 cm H; Med Associates ENV-515). On the exterior of the chamber, a strip of 16 X 16 infrared photocell beams placed 1 inch apart and 2.5 inches from the floor detected horizontal movement via beam breaks. The chamber was calibrated to enable measurement of distance traveled and center activity. A second row of 16 x 16 photosensors placed 4.5 inches from the floor tracked vertical movement of the mice. The photocells were interfaced to a PC computer running MedPC software for recording and analysis. Experiments were conducted under dim lighting conditions.

### Locomotor Activity

The locomotor activity experiment was conducted in a modified standard mouse chamber (L 30 cm x W 18 cm x H 18.5 cm) with the ventilation nozzle removed and a white card fixed over the area. Mouse chambers were placed inside



a white Plexiglas framebox measuring 33 cm x 20 cm x 19 cm made from white card and interfaced with a PC running ANY-maze software for recording and analysis of locomotor and rearing activity.

### Behavioral Procedures

Prior to all experiments mice were handled to acclimate to experimenter and experimental procedures.

#### Light-Dark Assay

The day prior to testing animals were acclimatized to the test room, weighed and tails were marked to facilitate identification by the experimenter. On test days, the experimental room was dark apart from the lighting required for the light-dark experiment. Mice weighed and acclimated to the lighting conditions for at least 1 hour prior to testing. The apparatus and dropping trays were thoroughly cleaned with 70% Novasan before each trial. Animals were administered i.p. injections of vehicle (0.9% sterile saline) or drug (DH $\beta$ E or nicotine) and were placed into the dark chamber, with the lid closed. Dependent variables included latency to enter the light chamber, defined as the time elapsed from the beginning of the experiment until the first entry was made in the light chamber, movements per second in each chamber with a locomotor movement unit defined as the two adjacent beam breaks (distance of 3 cm between light beams), entry into a chamber was defined as the subject breaking the second beam in the adjacent chamber (distance of 4.5 cm) and exploration of a chamber was defined as the subject breaking the first beam in the

adjacent chamber (distance of 1.5 cm). All data was broken down into 10 one-minute time bins. Behavior was assessed for ten minutes.

Dependent variables of latency, total movement and percentage of time spent in the light chamber were analyzed using a 3 way (genotype x age x test drug), between-subject ANOVA. Movements per second were analyzed using a repeated measure within-subject ANOVA with genotype and test drug as between-subject factors. Significant effects of time-bin were followed up by analysis using a further breakdown of the data. Tukey HSD *post hoc* tests evaluated main effects of DH $\beta$ E studies and LSD tests were used for *post hoc* analysis of the nicotine dose-response curves. Two-tailed *post hoc* t-tests were carried out where appropriate to assess significant interactions. The criterion for significance was set at  $p < 0.05$ .

### Marble burying test

Animals were habituated to the test room for at least 3 days prior to testing. Mice were weighed and had their tails marked with non-toxic indelible marker to expedite mouse identification during testing. On test day, animals were acclimated to the test room 2 to 3 hours prior to experimentation. All mice were weighed and returned to home cages. Animals were administered vehicle (saline) or drug (DH $\beta$ E or nicotine) i.p., and placed in a holding cage for 15 minutes. For testing, subjects were placed at the perimeter of the marble grid inside of marble burying apparatus. Mice were allowed to dig and explore the apparatus for the duration of the experimental session. For subjects administered DH $\beta$ E behavior was assessed for 30 minutes. For subjects administered nicotine behavior was for 15 minutes. Marbles at least  $\frac{1}{2}$  covered were defined as buried. A genotype x age x treatment,

between-subjects ANOVA was used to assess marbles buried. Tukey HSD *post hoc* tests evaluated main effects with more than one level for DH $\beta$ E studies and LSD tests evaluated significant effects for the nicotine dose response curves. Two-tailed *Post hoc* t-tests were carried out where appropriate to assess significant interactions. Criterion for significance was set at  $p < 0.05$ .

### Open-field test

On test days, animals were acclimated to a holding room adjacent to the test room for at least 30 minutes prior to testing. Animals were then transferred as a group in home cages to the test room at the time of testing. The open field apparatus was cleared of any debris and thoroughly cleaned with 70% ethanol before each trial. Unlike the marble-burying test and open-field, no drug was given during the open-field test. Mice were placed in the central-most quadrant of the illuminated open-field arena and allowed to freely explore the apparatus for a period of 10 minutes. Dependent variables of time spent in the center, time spent in the periphery (thigmotaxis), distance travelled in the center and vertical counts in the center were analyzed using a between-subjects ANOVA to assess differences between genotypes and age. The criterion for significance was set at  $p < 0.05$ .

### Locomotor activity

The day prior to test day animals were acclimatized to the experimental room, weighed and tails were marked with non-toxic indelible ink. On test days, the experimental room was dimly lit by two 23W n: vision fluorescent LED light bulb placed at 11 inches from the chambers. All animals were weighed and returned to

home cage and acclimated to the room for a period of 1 to 2 hours prior to testing. The locomotor apparatus was cleared of all debris and thoroughly cleaned with 70% Novasan before each trial. Animals were administered vehicle (saline) or drug (nicotine) i.p. and were placed into the apparatus for 10 minutes. Distance travelled, time immobile, wall rearing and time spent rearing were measured. A between-subject (2 x 7; genotype x nicotine treatment) ANOVA was used to assess significance of behavioral parameters, and the criterion for significance was set at  $p < 0.05$ . *Post hoc* LSD tests assessed significance between groups following main effects and two-tailed *Post hoc* t-tests were carried out where appropriate to assess significant interactions of drug treatment within a genotype.

## Results

### Assessing the contributions of the $\beta 2^*nAChRs$ on anxiety-like behavior

To assess contributions of high affinity  $\beta 2^*nAChRs$  to anxiety-like behavior, WT,  $\beta 2HET$  and  $\beta 2KO$  mice were tested in the light-dark, marble burying and open field assays. Prior to light-dark and marble burying assays, mice also received injection of saline vehicle or  $DH\beta E$ , a selective  $\beta 2^*nAChR$  antagonist.

#### Light-Dark Assay

There was no effect of genotype, age or  $DH\beta E$  treatment on mouse latencies to enter the light chamber or on the percentage of time that mice spent in the light chamber (Figure 1). For the measure of movements per second in the light chamber, however, there was a nearly significant interaction of genotype x age x treatment ( $F_{2, 47} = 3.131$ ,  $p = 0.053$ ). There was also a significant interaction of time bin x genotype x treatment for the measure of movements per second in the light chamber ( $F_{18, 423} = 2.017$ ,  $p = 0.008$ ; Figure 2). Subsequent analysis of the first and last 5 time bins returned a significant interaction of genotype x age x treatment on movements per second in the light chamber during the first 5 min ( $F_{2, 47} = 3.220$ ,  $p = 0.049$ ), but not during the last 5 min of the 10 min session (Figure 3). These effects were apparent in aged (24 month) but not middle aged (12 month) mice. *Post hoc* t-tests revealed that saline-injected, 24-month-old  $\beta 2KO$  mice showed a trend for greater exploration of the light chamber than saline-injected 24-month-old WT mice. Following  $DH\beta E$  injection, however, WT and  $\beta 2KO$  mice showed a similar level of exploration activity as measured by movements per second in the light chamber,

suggesting these effects were mediated by  $\beta 2^*nAChRs$ . It is interesting that a reduction of  $\beta 2^*nAChR$  expression in  $\beta 2HET$  mice appeared to result in the opposite effect following  $DH\beta E$  treatment in 24-month-old animals. *Post-hoc* t-tests revealed a significant difference between WT and  $\beta 2HET$  mice following  $DH\beta E$  injection ( $p = 0.03$ ). These effects were specific to the light chamber. There was no effect of genotype, age or treatment on movements per second in the dark chamber, suggesting that these observations were not due to non-specific effects of genotype, age or treatment on locomotor activity but were specific to exploration of the more aversive light chamber. Due to small n sizes in some of these groups, these preliminary data should be replicated (Table 1).

#### Marble Burying Task

There was a main effect of genotype ( $F_{2, 60} = 4.219$ ,  $p = 0.02$ ) and a non-significant trend for an interaction of genotype x age ( $F_{2, 60} = 2.974$ ,  $p = 0.06$ ) for number of marbles buried (Figure 4). *Post hoc* t-tests revealed that 12-month-old  $\beta 2HET$  mice buried significantly fewer marbles than their wild type counterparts ( $t_{13} = 3.507$ ,  $p = 0.004$ ), suggesting that a reduction in  $\beta 2^*nAChR$  expression promoted anxiolysis-like behavior in these mice. This was not observed when  $\beta 2^*nAChRs$  were completely absent, however; there was no significant difference in marble burying between WT and  $\beta 2KO$  mice at 12 months. There were also no differences between WT and  $\beta 2KO$  or  $\beta 2HET$  mice observed at 24 months of age ( $t$ 's  $< 1.0$ ) when WT subjects would be expected to show an overall reduction in the expression of their  $\beta 2^*nAChRs$  (S. W. Rogers, Gahring, Collins, & Marks, 1998).

## Open Field

There was a significant effect of genotype on distance traveled in the perimeter ( $F_{2, 93} = 3.664$ ,  $p = 0.03$ ) but not in the center of the open field test (Figure 5). Compared to WT subjects,  $\beta 2$ KO mice traveled a greater distance around the perimeter of the field ( $t_{70} = 1.95$ ,  $p = 0.055$ ). There was no difference between WT and  $\beta 2$ HET perimeter activity ( $t_{54} = 0.75$ ,  $p = 0.457$ ). There was also a significant effect of genotype on rearing activity as measured by vertical counts in the perimeter of the open field. *Post hoc* t-tests showed that  $\beta 2$ KO mice reared more than WT mice ( $t_{70} = 2.302$ ,  $p = 0.024$ ). There was no difference observed between WT and  $\beta 2$ HET mice. There was no effect of genotype on rearing activity in the center of the chamber or on total time spent in either the center or the perimeter of the open field ( $F$ 's  $< 1.0$ ). Together these data are consistent with studies, which suggest that  $\beta 2$ KO mice show elevated levels of exploration (Avale et al., 2008; King, Caldarone, & Picciotto, 2004).

### Assessing the contributions of the $\alpha 4^*$ nAChR on anxiety-like behavior

To assess contributions of high affinity  $\alpha 4^*$ nAChRs to anxiety-like behavior, WT,  $\alpha 4$ HET and  $\alpha 4$ KO mice were tested in the light-dark, marble burying and open field assays. Prior to light-dark and marble burying assays, mice also received injection of saline vehicle or DH $\beta$ E, a selective  $\beta 2^*$ nAChR antagonist.

High affinity  $\beta 2^*$ nAChRs assemble with the  $\alpha 4$  subunit in most areas of the brain.

Anxiety assays were performed in  $\alpha 4$ HET and  $\alpha 4$ KO mice to assess if the  $\alpha 4$  subunit contributes to anxiety-like behavior in the light-dark and marble burying task as well as to assess if a loss of function of the  $\alpha 4^*$ nAChRs would open field tasks.

### Light-Dark

There was a main effect of genotype ( $F_{2, 74} = 3.341$ ,  $p = 0.038$ ) and a main effect of treatment ( $F_{2, 74} = 6.466$ ,  $p = 0.013$ ) for latency to enter the bright chamber (Figure 6).  $\alpha 4$ KO mice displayed longer latencies to enter the light chamber compared to WT mice ( $p = 0.03$ ), suggesting that absence of this subunit supported an anxiogenic-like phenotype. Partial deletion of the  $\alpha 4^*$ nAChRs was not sufficient for expression of this phenotype, there was no difference in latency between WT and  $\alpha 4$ HET mice. Similarly, antagonism of  $\beta 2^*$ nAChRs with DH $\beta$ E resulted in an elevation in latency, suggesting that blockade of  $\alpha 4\beta 2^*$ nAChRs might support anxiogenesis in female mice. There was also a significant effect of genotype ( $F_{2, 74} = 4.311$ ,  $p = 0.017$ ) and treatment ( $F_{1, 74} = 4.009$ ,  $p = 0.049$ ) for percentage of time spent in the light chamber (Figure 7). As with latency measures,  $\alpha 4$ KO mice but not  $\alpha 4$ HETs spent significantly less time in the light chamber than WT mice ( $p = 0.008$ ) and DH $\beta$ E injection led to a significant reduction in time spent in the light chamber. There was a nearly significant main effect of genotype for movements per second in the light chamber ( $F_{2, 74} = 3.060$ ,  $p = 0.053$ ) and a significant main effect of genotype on movements per second in the dark chamber ( $F_{2, 74} = 4.755$ ,  $p = 0.011$ ). *Post hoc* tests indicated that  $\alpha 4$ KO but not  $\alpha 4$ HET mice showed a trend for less exploration than WT mice in the light chamber ( $p = 0.09$ ). This was in the opposite direction of dark chamber exploration activity where  $\alpha 4$ KO mice showed a significant elevation in movements per second compared to WT mice ( $p = 0.006$ ). Overall these data support previous findings suggesting that  $\alpha 4^*$ nAChRs regulate anxiety-like behavior.



Due to small n sizes in some groups, however, these data should be replicated (Table 1).

### Marble Burying

There was a main effect of age ( $F_{1, 74} = 9.428, p = 0.003$ ) on marble burying behavior indicating that aged mice buried overall significantly fewer marbles than middle aged animals (Figure 9). Although main effects for genotype and DH $\beta$ E treatment failed to reach significance, there was a significant interaction of genotype and treatment ( $F_{2, 74} = 3.414, p = 0.038$ ). DH $\beta$ E injection led to a significant reduction of marbles buried in  $\alpha$ 4HET mice ( $p < 0.007$ ) but not in WT mice. It is possible that  $\alpha$ 4HET mice were more responsive to the antagonist due to a reduction in their basal level of  $\alpha$ 4 $\beta$ 2\*nAChRs or in the shift of the configuration of these receptors to the  $\alpha$ 4 $\beta$ 2 $\beta$ 3 stoichiometry. In contrast, there was no effect of DH $\beta$ E treatment on marble burying in  $\alpha$ 4KO mice, supporting the hypothesis that this effect was driven by receptors that contain  $\alpha$ 4 and  $\beta$ 2 subunits, i.e.  $\alpha$ 4 $\beta$ 2\*nAChRs. Drugs used to treat anxiety in humans have been found to reduce the number of marbles buried this task (Nicolas, Kolb, & Prinssen, 2006) these results suggest that inhibition of  $\alpha$ 4 $\beta$ 2\*nAChRs may promote anxiolysis.

### Open Field

There was no effect of genotype and no interaction of genotype with age on any measure in the open field study. WT,  $\alpha$ 4HET and  $\alpha$ 4KO mice spent a similar amount of time in the perimeter and the center of the open arena and did not differ in their rearing activity in the center or the perimeter of the field (Figure 10). This is in

contrast to the observation above that  $\beta 2$ KO mice spent significantly more time in exploration of the perimeter of the maze, suggesting that a stoichiometry other than  $\alpha 4\beta 2^*nAChRs$ , perhaps  $\alpha 6\beta 2^*nAChRs$ , might regulate exploration in an open field. There was a significant main effect of age for time spent in the center of the open field ( $F_{2, 111} = 3.283$ ,  $p = 0.041$ ), rearing in the center ( $F_{2, 111} = 4.240$ ,  $p = 0.017$ ), and rearing in the perimeter ( $F_{2, 111} = 17.250$ ,  $p < 0.001$ ) of the open field. *Post-hoc* tests revealed that 12-month-old mice spent more time than 24-month-olds in the center of the field ( $p = 0.053$ ). 6-month-old mice did not differ from the other age groups. 12-month-old mice showed significantly greater rearing activity in the center of the field than 6-month-old mice ( $p = 0.024$ ). This was likely due to 6-month-old mice spending less time in the center of the field given that they showed a trend for more rearing than 12-month-olds ( $p = 0.072$ ) and significantly more rearing activity than 24-month-olds ( $p < 0.001$ ) in the perimeter of the open field.

#### Assessing the contributions of $\alpha 6^*nAChR$ on anxiety-like behavior

To assess contributions of high affinity  $\alpha 6^*nAChRs$  to anxiety-like behavior, WT and a gain of function  $\alpha 6L9S$  mice were tested in the light-dark, marble burying and locomotor activity assays (Table 4). Prior to light-dark and marble burying assays, mice also received injection of saline vehicle or nicotine.

Although most  $\beta 2^*nAChRs$  assemble with  $\alpha 4$ , within the mesolimbic and catecholaminergic projection pathways, the high affinity  $\beta 2^*nAChRs$  assemble with  $\alpha 6$  subunits to make  $\alpha 6\beta 2\beta 3nAChRs$  and  $\alpha 6\alpha 4\beta 2\beta 3nAChRs$ .  $\alpha 6L9S$  gain of function mice were compared to WT mice to assess if activation of  $\alpha 6\beta 2^*nAChRs$  supports anxiety-like behavior in the light-dark and marble burying tasks in the presence or

absence of nicotine (0, 0.001, 0.0032, 0.01, 0.032, 0.1, 0.32 mg/kg i.p.). Since previous studies have shown that male L9S mice show a hyper-locomotor response to nicotine, we also assessed female mice in a locomotor assay in the presence or absence of nicotine.

### Light-Dark

There was a main effect of nicotine dose for measures latency ( $F_{6, 70} = 8.278$ ,  $p < 0.001$ ) (Figure 11) and percent time spent in the light chamber ( $F_{6, 70} = 3.703$ ,  $p = 0.003$ ) (Figure 12). Consistent with previous studies showing that low doses of nicotine are anxiolytic and high doses are anxiogenic, *post-hoc* analysis revealed that mice receiving 0.32 mg/kg i.p. nicotine showed a significant increase in latency and low doses of nicotine (0.01, 0.032, and 0.1 mg/kg i.p.) resulted in significant decreases in latency to enter the light chamber compared to saline-injected control mice. Low doses of nicotine (0.01, 0.032, and 0.1 mg/kg i.p.) also resulted in significant elevations in the percentage of time that mice spent in the brightly lit chamber. This behavior was similar for WT and L9S mice, there was no effect of genotype on latency or time spent in the light chamber. There was also an effect of nicotine dose on light and dark chamber exploration as measured by movements per second in the light ( $F_{6, 70} = 4.378$ ,  $p = 0.001$ ) and dark ( $F_{6, 70} = 3.883$ ,  $p = 0.002$ ) chambers. There was a significant interaction of time bin with genotype in movements per second in the light chamber ( $F_{9, 360} = 2.007$ ,  $p = 0.036$ ) and a significant interaction of genotype with nicotine dose on movements per second in the dark chamber ( $F_{6, 70} = 2.475$ ,  $p = 0.031$ ). WT mice showed an inverted U pattern of the movements per second measure in the light and dark chambers. Low doses of nicotine (0.01, 0.032 and 0.1 mg/kg) stimulated exploration and the high 0.32 mg/kg

i.p. dose of nicotine attenuated exploration in the WT mice ( $p$ 's < 0.05). L9S mice, however, did not show a decline in light or dark chamber exploration at the higher doses.

### Marble Burying

There was a main effect of genotype ( $F_{6, 70} = 3.246$ ,  $p = 0.007$ ) for marbles buried. Compared to WT mice,  $\alpha 6$ L9S mice showed a significant decrease in digging activity, suggesting that activation of the  $\alpha 6\beta 2$ \*nAChRs promoted anxiolysis-like behavior in this task. There was also a main effect of dose for the measure of marbles buried ( $F_{1, 70} = 19.768$ ,  $p < 0.001$ ) with the highest dose of nicotine resulting in a significant decrease in the number of marbles buried ( $p = 0.03$ ). Marble burying data are shown in Figure 14.

### Locomotor Activity

There was no effect of nicotine dose on locomotor activity behavior as measured by distance traveled or time spent immobile in the apparatus under low-light conditions ( $F$ 's < 1.0). Unlike their male counterparts that show hyperlocomotor responses to nicotine (Champtiaux et al., 2002; Champtiaux et al., 2003; Cui et al., 2003; Drenan et al., 2008; Drenan et al., 2010), female  $\alpha 6$ L9S mice did not show a leftward shift in distance traveled following nicotine injection. There was, however, a significant effect of genotype on time spent rearing with  $\alpha 6$ L9S mice showing an elevation in this behavior compared to WT mice ( $F_{1, 136} = 3.934$ ,  $p = 0.049$ ). There was also a significant effect of nicotine dose on number of rearing events that was independent of genotype ( $F_{3, 136} = 4.280$ ,  $p = 0.006$ ). The 0.032 mg/kg i.p. dose of

nicotine resulted in an increase in rearing activity compared to when mice had received saline injections.

## Discussion

The main findings of this thesis work are that  $\beta 2$ HET and  $\alpha 4$ HET mice showed increased sensitivity to the antagonist di-hydrobeta-erythroidine (DH $\beta$ E). Consistent with previous studies  $\alpha 4$ KO mice showed an anxiogenic-like phenotype compared to WT mice. Conversely, whilst  $\beta 2$ KO mice failed to show significant changes in anxiety-like behavior compared to WT mice, these mice did show increased locomotor activity and exploratory behavior in the perimeter of the open field test, which is consistent with previous reports. Also, treatment with low doses of nicotine (0.01, 0.032 and 0.1 mg/kg) produced anxiolytic-like effects in WT and  $\alpha 6$ L9S mice, whilst a high dose of nicotine produced anxiogenic-like effects in WT and  $\alpha 6$ L9S mice. Activation of the  $\alpha 6^*$ nAChRs supports an anxiolytic-like phenotype in the light-dark assay and marble burying test. Unlike male  $\alpha 6$ L9S mice, female  $\alpha 6$ L9S mice did not show hyperlocomotor responses to nicotine. However, female  $\alpha 6$ L9S mice did appear to be less sensitive than WT mice to locomotor suppressant effects of a high (0.32 mg/kg) dose of nicotine in the dark chamber.

The nAChRs containing the  $\alpha 4$  and  $\beta 2$  nAChR subunits are the most abundantly expressed nAChRs in the central nervous system. These receptor subtypes are expressed in a region specific manner in the brain and those containing the  $\beta 2$  are the most abundant in the brain (McGehee & Role, 1995). The  $\alpha 4\beta 2^*$ nAChRs have also been found to play a role in the anxiolytic effect of nicotine (Besson, Suarez, Cormier, Changeux, & Granon, 2008; King, Caldarone, & Picciotto,

2002; McGranahan, Patzlaff, Grady, Heinemann, & Booker, 2011). Preliminary studies investigating the contributions of the  $\beta 2$  nAChR subunit shows that elimination of the  $\beta 2$  subunit does not promote anxiolytic-like behavior in the open field test. There was no effect of genotype or age on center activity in the open field test. However,  $\beta 2$ KO mice showed increased levels of exploration in the perimeter of the open field. These results may be due to small n sizes used in these studies (Table 1), interpretation of these studies would benefit from replication. Although a null mutation for the  $\beta 2$  subunit did not produce an anxiety-like phenotype, in these studies, a loss of the  $\alpha 4$  subunit promoted anxiogenic-like behavior. Consistent with previous studies using the elevated-plus maze (Ross et al., 2000)  $\alpha 4$  Knockout ( $\alpha 4$ KO) female mice show increased anxiety-like behavior as shown by increased latency to enter the light chamber compared to heterozygous mice, decreased percentage of time spent in the light chamber and decreased movements per second in the light chamber, compared to WT mice in the light-dark assay. Taken together, these results suggest that  $\alpha 4\beta 2$ \*nAChRs and possibly non- $\beta 2$ \*nAChRs such as  $\alpha 4\beta 3$  nAChRs regulate anxiety-like behavior in female mice. Further experiments are needed to determine the stoichiometry of the receptors involved in the behaviors observed.

In contrast to  $\alpha 4$ KO mice, studies in  $\alpha 6$ L9S mice revealed that stimulation of  $\alpha 6\beta 2$ \*nAChRs receptors might promote anxiolysis.  $\alpha 6$ L9S mice spent more time rearing than WT mice in the open field, buried fewer marbles in a novel open chamber, and spent more time than WT mice in exploration of the light chamber, but not the dark chamber in the light dark assay. Together these findings suggest that activation of the  $\alpha 6$ \*nAChRs may attenuate anxiety phenotype and promote

anxiolysis-like behavior in rodents. Taken together, these results suggest that  $\alpha 4\beta 2^*$ nAChRs and  $\alpha 6\beta 2^*$ nAChRs, possibly  $\alpha 4\alpha 6\beta 2^*$ nAChRs regulate anxiety-like behavior in female mice. Further experimentation in  $\alpha 6L9S$  mice crossed to  $\alpha 4KO$  mice could help to clarify the stoichiometry of these receptors and will establish if activation or inhibition of subtypes of  $\alpha 4\alpha 6\beta 2^*$ nAChRs supports anxiolysis in females.

Although genetic approaches are a useful method for assessing the role of genes on behavioral phenotypes, caution is needed when interpreting results. An important factor to consider is that alteration or deletion of a gene leads to compensatory mechanisms that are present over the lifetime of the animal. This makes it difficult to determine whether the effects observed in genetically modified mice are due the alteration of a gene, or if they are due to compensatory changes that occur during development. Another factor to consider is that knockout mice can pinpoint the genes that mediate the effects of a drug (e.g. nicotine). However, the deletion of a gene does not reveal whether variability in this gene can alter sensitivity to the drug investigated.

Another method for assessing the role of nAChRs in anxiety-like behavior involves pharmacological inactivation of the  $\beta 2^*$ nAChR with the selective antagonist, DH $\beta$ E. DH $\beta$ E acts on  $\beta 2^*$ nAChRs that co-assemble with other subunits to form  $\alpha 4\beta 2$ ,  $\alpha 6\alpha 4\beta 2\beta 3$ ,  $\alpha 6\beta 2\beta 3$  and  $\alpha 4\alpha 5\beta 2$ nAChRs. Research has shown that DH $\beta$ E blocks nicotine-mediated release of neurotransmitters (S. S. Khiroug, Khiroug, & Yakel, 2004; Lu et al., 1998; M. J. Marks et al., 2000; Whiteaker et al., 2000) and nicotine-elicited responses on spontaneous inhibitory postsynaptic current (IPSC) frequency and excitatory postsynaptic current (EPSC) in brain slices (Alkondon &



Albuquerque, 2002; Mansvelder, Keath, & McGehee, 2002). Therefore, the inactivation of  $\beta 2^*$ nAChRs by DH $\beta$ E is a useful pharmacological tool to investigate the role  $\beta 2^*$ nAChRs play in behaviors such as anxiety that are regulated by multiple neurotransmitter systems. Our lab has shown that antagonism of the  $\beta 2^*$  nAChRs with the selective antagonist DH $\beta$ E supports anxiolytic-like behavior in male C57BL/6 mice (unpublished). These findings suggest that inactivation of these high affinity receptors in male mice is anxiolytic. This is the opposite of what we observed in C57BL/6 females in most of the present studies.

One of the findings from these studies is that effects of DH $\beta$ E on anxiety-like behavior in female mice appear to be age and genotype dependent. Rogers and colleagues have shown that aged CBAJ mice (24-28 months old) have a reduction in the expression of  $\alpha 4\beta 2^*$ nAChRs in key cholinergic regions in the brain (S. W. Rogers, Gahring, Collins, & Marks, 1998). In these studies we report that aged  $\alpha 4^*$ nAChRs mice compared to younger mice showed decreased exploratory behavior and locomotor activity in the open field test. Given previous reports that the expression of the  $\alpha 4\beta 2^*$ nAChRs changes with age, we would expect to see changes in the anxiety phenotype in aged mice, however, age did not significantly affect anxiety-like behavior in the open field test or light-dark assay. It should be noted that our age groups defined in these studies did not include young adult mice. Interpretations of these data could benefit from the inclusion of mice which are younger to further assess the contributions of the  $\beta 2^*$ nAChRs on anxiety-like behavior across the life span in female mice.  $\beta 2$ HET aged mice were more sensitive than WT aged mice to anxiogenic-like effects of DH $\beta$ E treatment in the light-dark assay. These results further suggests that in aged mice which have a reduced

number of receptors due to normal aging (S. W. Rogers, Gahring, Collins, & Marks, 1998), the further reduction of the receptors via genetic manipulation and subsequent inactivation of the receptors with DH $\beta$ E potentiates anxiogenic-like behavior in aged heterozygous animals. These preliminary studies will benefit from replication using a more complete dose response curve for DH $\beta$ E.

In the open field test aged mice showed decreased exploratory behavior and locomotor activity compared to younger mice. Taken together,  $\beta$ 2HET and  $\alpha$ 4HET mice show increased sensitivities to DH $\beta$ E treatment, perhaps due to a decrease in  $\alpha$ 4\* and  $\beta$ 2\*nAChR expression. This finding suggests that inhibition of  $\beta$ 2\*nAChRs promotes anxiogenesis in female mice. Further investigation into the relative expression of  $\beta$ 2\*nAChRs in the brain would further elucidate the contributions of region specific changes in the expression of  $\beta$ 2\*nAChRs in aged and middle aged mice on anxiety-like behavior. It is important to note that in the marble burying test,  $\alpha$ 4HET mice treated with DH $\beta$ E showed anxiolytic-like effects compared to WT mice as measured by decreased marbles buried. Although more investigation is warranted, these studies overall, suggest that high affinity  $\beta$ 2\*nAChRs may be a useful therapeutic target for the treatment of anxiety disorders in elderly women.

In the marble burying test however, aged mice had decreased anxiety-like behavior compared to middle aged mice. Aged mice buried less marbles than did middle aged mice. Results from the light-dark assay and open field test were not consistent in the marble burying test. Although the marble burying test provides predictive validity through the use of anxiolytics, many believe that this test does not accurately measure anxiety-like behavior but is a better model for compulsive disorders (Jimenez-Gomez, Osentoski, & Woods, 2011; Witkin, 2008). The lack of

internal controls in the marble burying test makes it less effective at assessing anxiety-like behavior in rodents compared to the light-dark assay which possesses its own internal controls. Taken together, it is likely that for our experimental paradigm the marble burying test is not an effective method for assessing anxiety-like behavior in female mice. To further assess anxiety-like behavior which is not locomotor dependent, the use of other mouse models of anxiety such as the conditioned emotional response assay and the potentiated acoustic startle paradigms would be more suitable tests as they include behavioral controls to assess if changes in anxiety-like behavior are learning-dependent (Davis, 1990).

Animal research has shown that nicotine's activity at nAChRs is dose dependent and doses of nicotine produces dissociate effect where low doses of nicotine promote anxiolytic-like effects and high doses produce anxiogenic-like effects on behavior (J. P. Changeux et al., 1998; File, Kenny, & Ouagazzal, 1998; File, Kenny, & Cheeta, 2000; Matta et al., 2007; M. R. Picciotto, Brunzell, & Caldarone, 2002; M. R. Picciotto, 2003; M. R. Picciotto, Addy, Mineur, & Brunzell, 2008; Tucci, Cheeta, Genn, Seth, & File, 2002; Tucci, Cheeta, Seth, & File, 2003; Viveros, Marco, & File, 2006). Consistent with previous studies, effects of nicotine on anxiety-like behavior were dose-dependent with low doses of nicotine decreasing anxiety-like behavior as measured by decreased latencies to enter the light chamber and an increase in the percentage of time mice spent in that chamber and increased exploration of mice while in the light chamber. In contrast, the high dose of nicotine produced anxiogenic-like effects, increasing latencies to enter the light chamber, decreasing time spent in the light chamber, and decreasing movements per second in the light but not the dark chamber of the light-dark assay. Drenan and colleagues (2008) showed that male  $\alpha 6L9S$  mice exhibited hyperlocomotor responses to

nicotine when compared to WT mice.  $\alpha 6\beta 2^*$ nAChRs have a highly selective pattern of expression in catecholaminergic neurons and activation of these receptor subtypes is necessary for nicotine's psychostimulant effects and administration. In these studies, in contrast to male  $\alpha 6L9S$  mice, female  $\alpha 6L9S$  did not show an overall hyperlocomotor response to nicotine when compared to WT mice in the locomotor activity task, but did appear to be less sensitive than WT mice to locomotor suppressant effects of a high (0.32 mg/kg) dose of nicotine in the dark chamber. Together these findings suggest that activation of the  $\alpha 6^*$ nAChRs may promote anxiolysis. An extended dose-response curve for nicotine locomotor effects will help establish if activation of  $\alpha 6^*$ nAChRs also attenuates anxiogenic-like effects of nicotine.

### Summary

These studies are among the first to assess the contributions of high affinity nAChRs on anxiety-like behavior in female mice. Consistent with previous studies performed mainly in male mice, low doses of nicotine promoted anxiolysis-like behavior and high dose nicotine promoted anxiogenic-like behavior in the light-dark assay. Although  $\alpha 6L9S$  mice showed a consistent behavioral phenotype across all three behavioral measures (light-dark, marble burying and open field), the effects of nicotine and other genotypic effects did not carry-over to the marble burying test, suggesting that the marble burying test may not be an appropriate measure for assessing anxiety-like behavior in female mice in our experimental paradigms. Decrease in  $\alpha 4^*$  and  $\beta 2^*$ nAChR expression caused increased sensitivities to DH $\beta$ E treatment suggesting that inhibition of  $\beta 2^*$ nAChRs promotes anxiogenesis in female

mice. Consistent with previous studies using the elevated plus maze,  $\alpha 4$ KO female mice displayed an anxiogenic-like phenotype in the light-dark assay. The selective  $\beta 2^*$ nAChR antagonist DH $\beta$ E also enhanced anxiogenic-like behavior for measures in this task, suggesting that inactivation of  $\alpha 4^*$ nAChRs promotes anxiety-like behavior. In contrast, gain of function  $\alpha 6$ L9S mice showed an anxiolysis-like phenotype in the light-dark, locomotor, and marble burying tasks, suggesting that activation of  $\alpha 6\beta 2^*$ nAChRs promotes anxiolysis-like behavior in these tasks.

The effects observed in these studies may be mediated by sex hormones, however, it is currently difficult to accurately assay serum estradiol levels in female mice and traditional methods for carrying out vaginal smears often induce estrus. Therefore, without more effective methods for the assessment of hormone levels in female mice we cannot infer the contributions of hormones on anxiety-like behavior in our experimental paradigm.

Overall, these results provide evidence that  $\alpha 4\beta 2^*$ nAChRs and  $\alpha 6\beta 2^*$ nAChRs regulate anxiety-like behavior in female mice. Further experimentation will help to clarify the stoichiometry of these receptors and will establish if activation or inhibition of subtypes of  $\beta 2^*$ nAChRs supports anxiolysis in females. These experiments provide possible therapeutic targets for the treatment of anxiety disorders in women.

### Future Directions

It is important to consider the intricate interplay of regionally specific nAChR expression, the nature of the specific behavioral test used, and the physiological responses to identify genetic contributions on anxiety-like behavior. To further assess the neuroanatomical changes involved in the changes in anxiety-like

behavior observed in these studies, identification and quantification of the nAChRs subtypes present in key brain regions associated with anxiety would aid in elucidating the molecular changes associated with the age-dependent effects on behavior. Pending the availability of selective antibodies as tools, Western Blot analysis will provide information on the molecular changes in our experimental paradigm. To further elucidate which subtype of high affinity nAChRs are involved in anxiety-like behavior, the use of genetically manipulated mice strains such as crosses of  $\alpha 4$ KO mice with  $\alpha 6$ L9S mice or assessing the behavior of  $\alpha 6$ KO mice would further allow investigation of receptor stoichiometry involved in the behaviors found in these studies.

## Tables

**Table 1.** n sizes for mice in genotypic studies of anxiety-like behavior.

Behavioral test		Light-dark		Marble burying	
Genotype	Treatment	Age			
		12	24	12	24
WT ( $\beta$ 2)	SAL	5	6	4	6
	DH $\beta$ E	4	6	5	6
$\beta$ 2HET	SAL	2	4	3	3
	DH $\beta$ E	3	4	3	4
$\beta$ 2KO	SAL	5	7	6	7
	DH $\beta$ E	6	8	6	8
WT ( $\alpha$ 4)	SAL	9	7	9	8
	DH $\beta$ E	9	10	9	9
$\alpha$ 4HET	SAL	10	4	10	5
	DH $\beta$ E	9	3	10	3
$\alpha$ 4KO	SAL	9	3	9	2
	DH $\beta$ E	9	3	9	3

Treatment groups include saline (SAL) and di-hydrobeta-erythroidine (DH $\beta$ E). Genotypes include  $\beta 2$  knockout ( $\beta 2$ KO),  $\beta 2$  heterozygous ( $\beta 2$ HET), and wild type littermates (WT ( $\beta 2$ )),  $\alpha 4$  knockout ( $\alpha 4$ KO),  $\alpha 4$  heterozygous ( $\alpha 4$ HET) and wild type littermates (WT ( $\alpha 4$ )).

**Table 2.** Open field experiment n sizes for wild type (WT),  $\beta 2$  heterozygous ( $\beta 2$ HET) and  $\beta 2$  knockout ( $\beta 2$ KO) mice.

Behavioral test	Open field	
Genotype	Age	
	12	24
WT	15	19
$\beta 2$ HET	11	11
$\beta 2$ KO	18	20



**Table 3.** Open field experiment n sizes for wild type (WT),  $\alpha 4$  heterozygous ( $\alpha 4$ HET) and  $\alpha 4$  knockout ( $\alpha 4$ KO) mice.

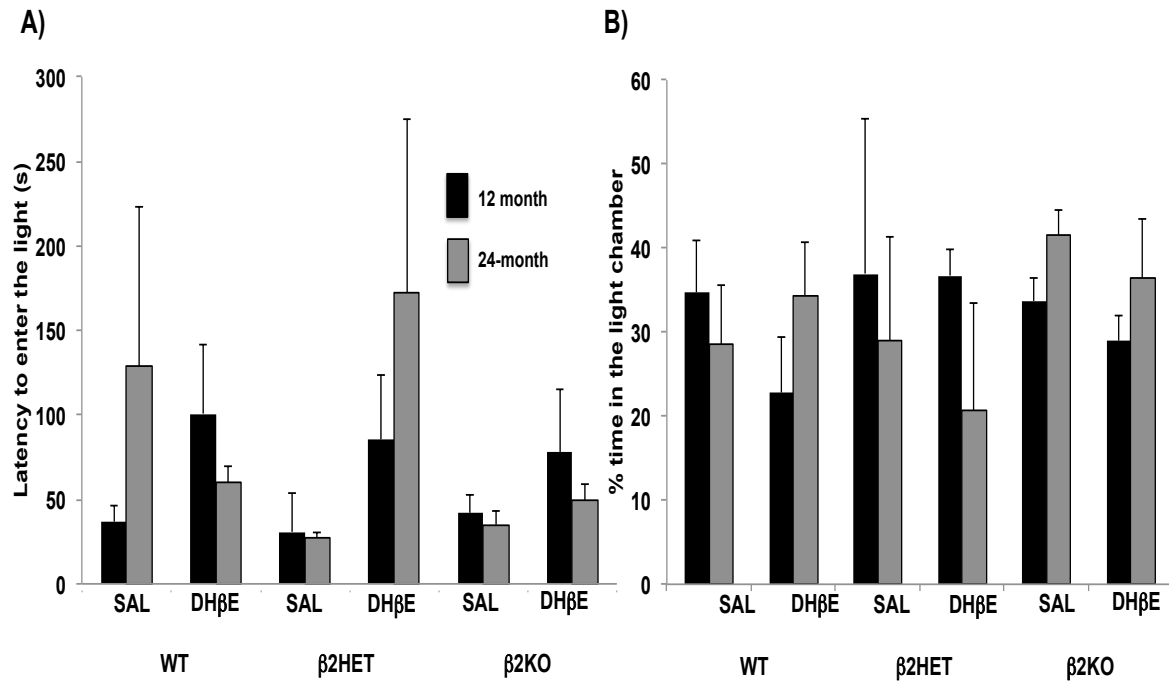
Behavioral test	Open field		
Genotype	Age		
	6	12	24
WT	11	15	11
$\alpha 4$ HET	13	14	14
$\alpha 4$ KO	12	19	11

**Table 4.** Average age in months for wild type (WT) and  $\alpha 6$ L9S (L9S) transgenic mice.

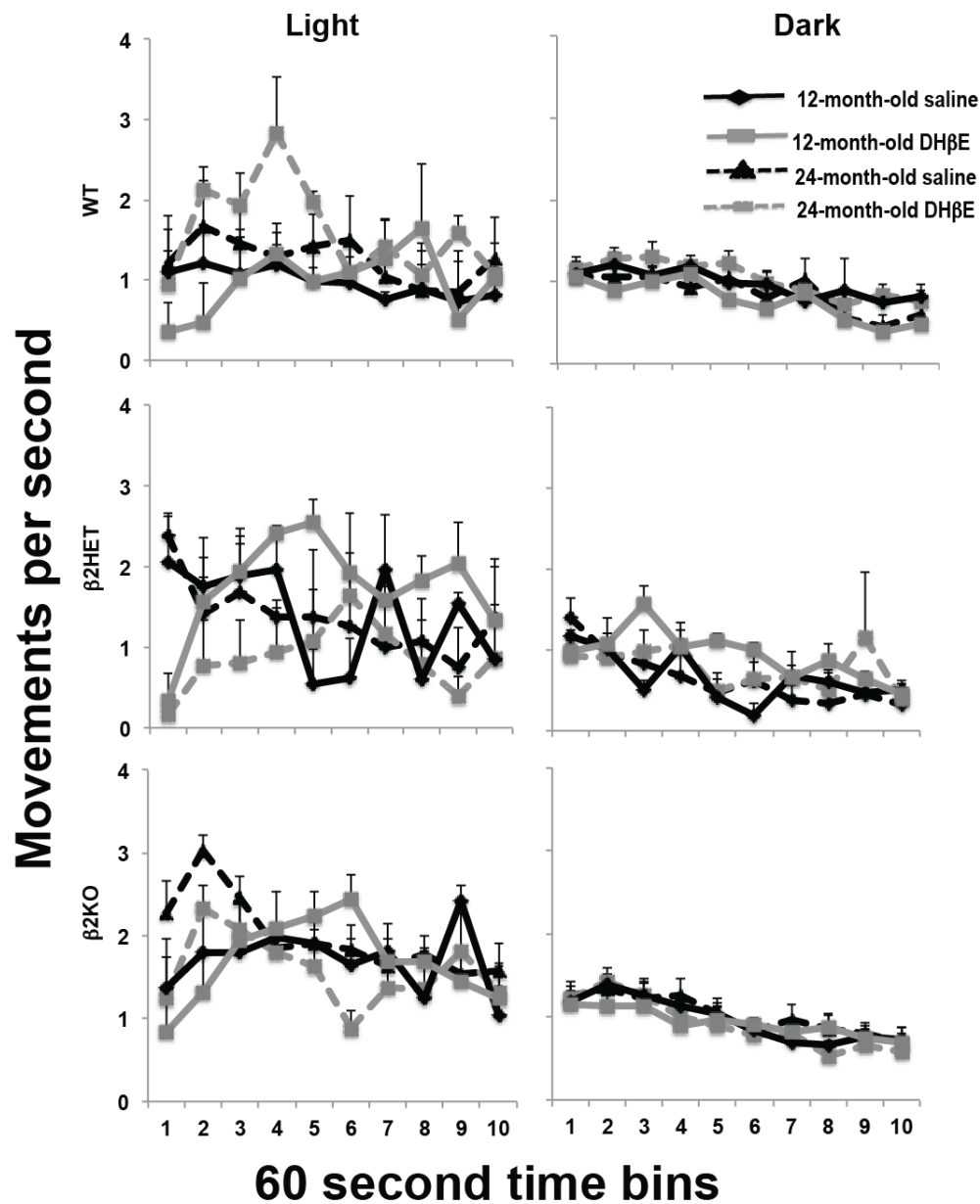
Genotype	Nicotine Dose (mg/kg; i.p.)						
	0	0.001	0.0032	0.01	0.032	0.1	0.32
WT	8.1±1 (6)	7.9±0.8 (6)	7.8±0.5 (6)	8.0±0.9 (6)	8.1±1 (6)	7.9±1 (6)	7.9±0.9 (6)
L9S	7.6±0.7 (6)	8.2±0.7 (6)	7.7±0.5 (6)	6.2±0.6 (6)	7.0±0.6 (6)	6.6±0.4 (6)	7.4±0.7 (6)

Data are represented as Mean  $\pm$  SEM; n sizes are depicted in parenthesis.

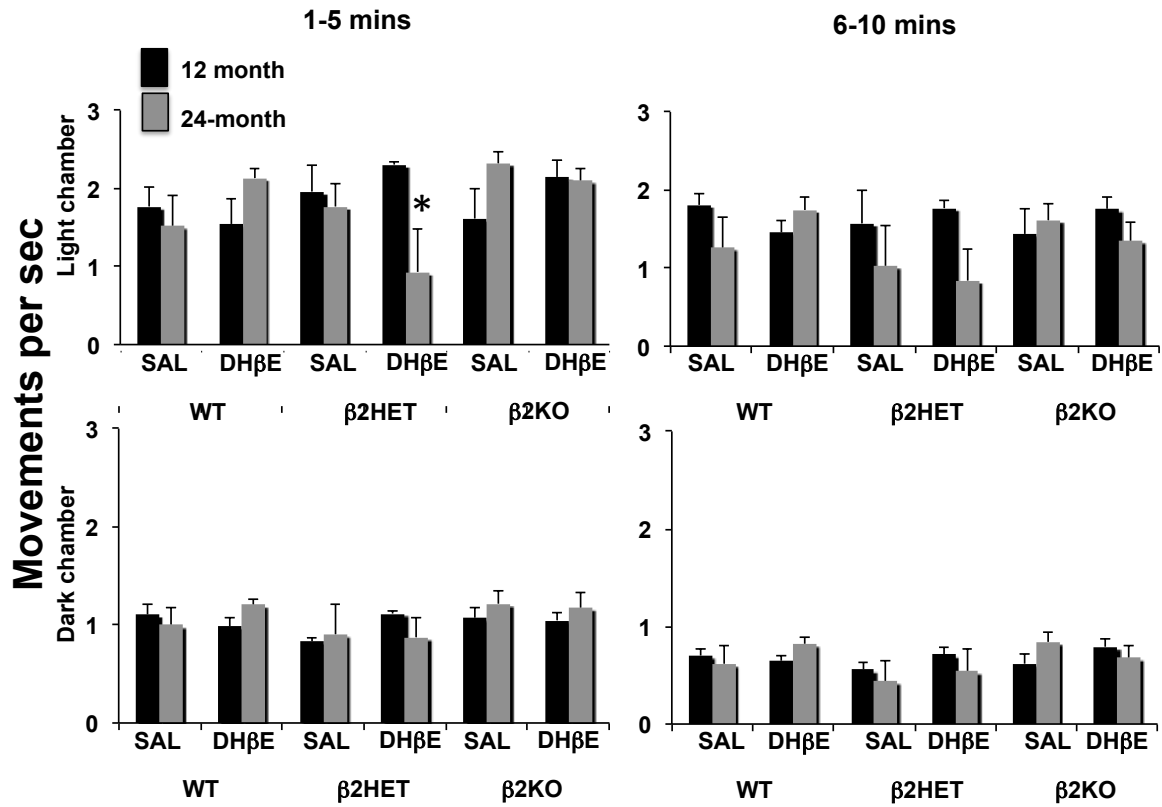
## Figures



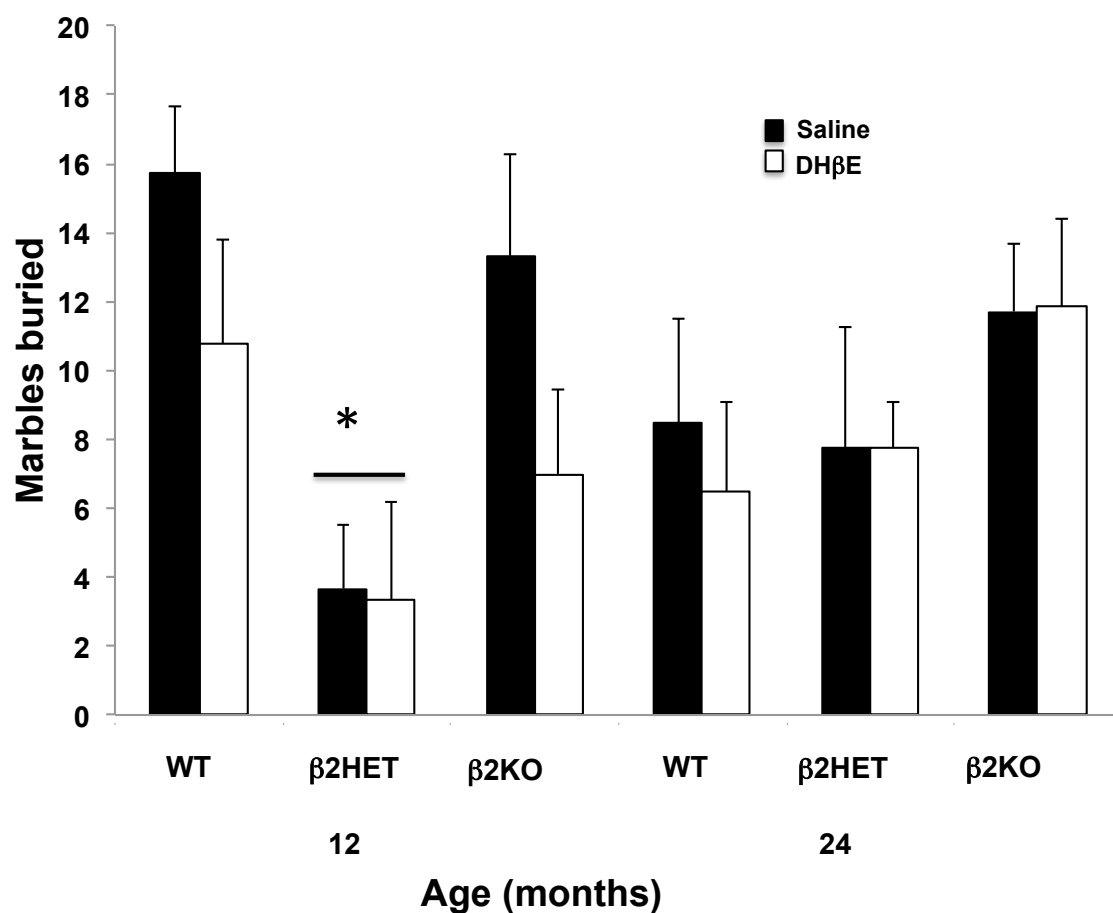
**Figure 1.** Light- dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\beta 2^*nAChR$  knockout mice ( $\beta 2KO$ ) and  $\beta 2^*nAChR$  heterozygous mice ( $\beta 2HET$ ) on measures of latency to enter the light chamber and percentage of time spent in the light chamber. There was no effect of genotype or di-hydrobeta-erythroidine (DHβE) treatment or age or interaction of genotype by age by treatment on measures of light-dark activity. Data are expressed as mean  $\pm$  SEM; (n = 2-8 per group).



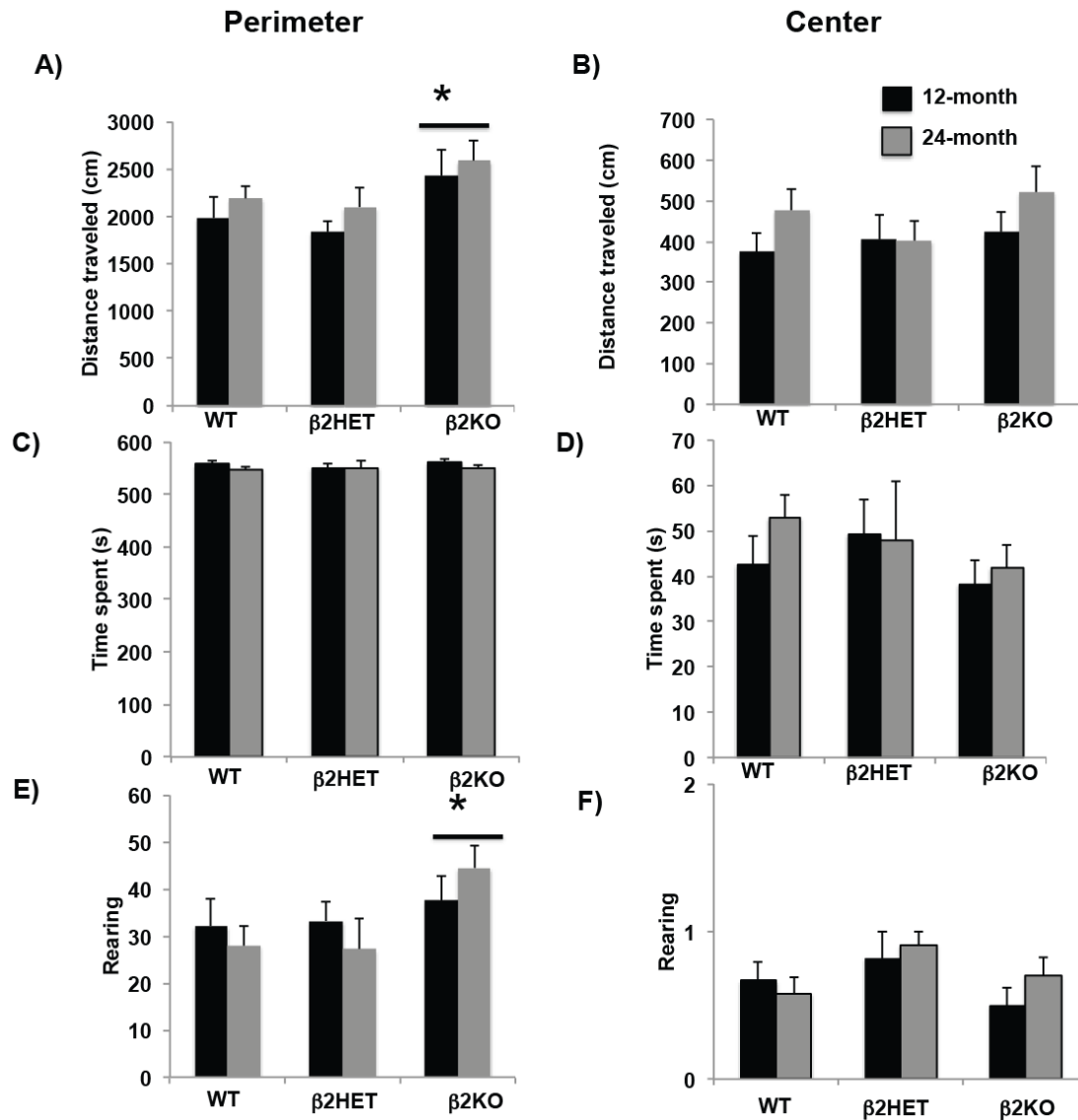
**Figure 2.** Light-dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\beta 2^*nAChR$  knockout mice ( $\beta 2KO$ ) and  $\beta 2^*nAChR$  heterozygous mice ( $\beta 2HET$ ) on measures of movement per second in the light and dark chambers. There was a significant effect of time bin by genotype by di-hydrobeta-erythroidine (DH $\beta$ E) treatment in the light chamber of the light-dark assay. No effect of genotype, treatment or age or an interaction of genotype by age by treatment on movements per second in the dark chamber. Data expressed as mean  $\pm$  SEM;  $p = 0.008$  ( $n = 2-8$  per group).



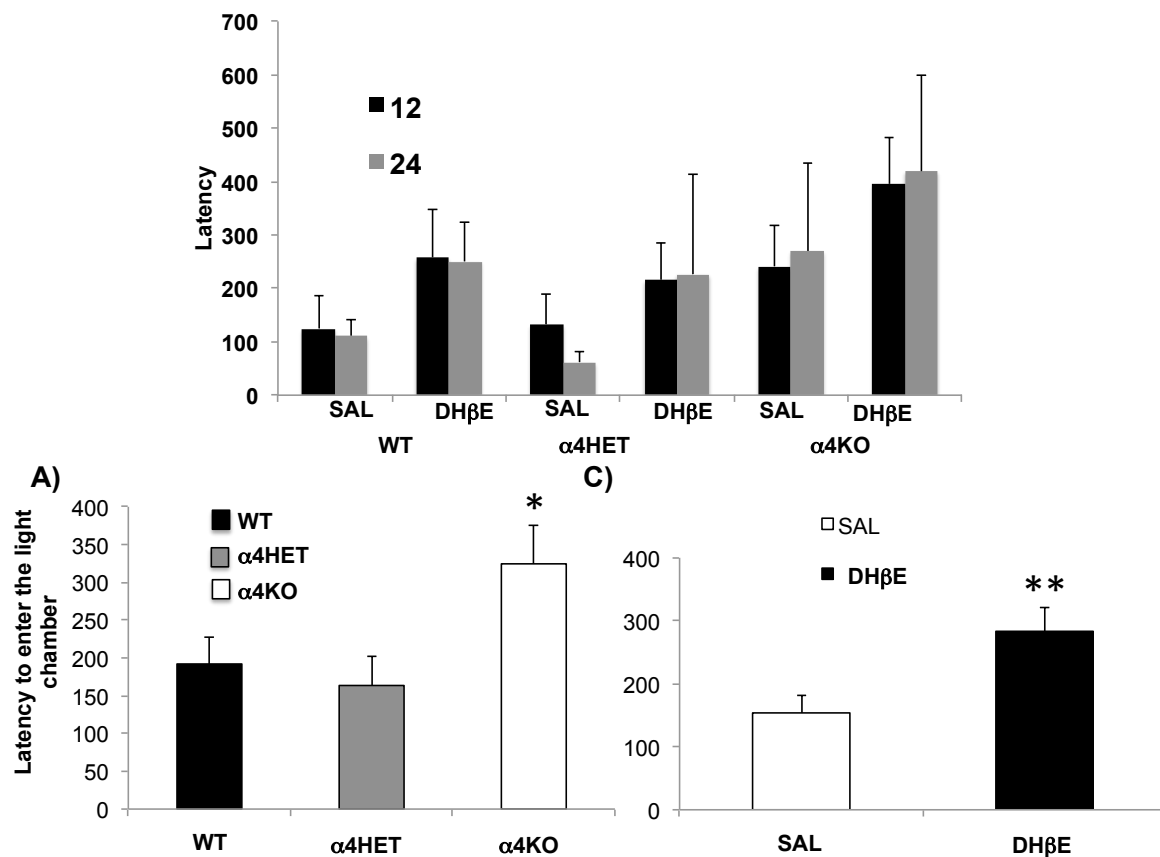
**Figure 3.** Light-dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\beta 2^*$ nAChR knockout mice ( $\beta 2$ KO) and  $\beta 2^*$ nAChR heterozygous mice ( $\beta 2$ HET) on measures of movement per second in the light and dark chambers. 24-month-old  $\beta 2$ HET mice treated with di-hydro-beta-erythroidine (DH $\beta$ E) had significantly reduced movement/sec in the light chamber at 1-5 minutes compared to 24-month-old WT mice treated with DH $\beta$ E. No significant effect of genotype, treatment or age was found in the dark chamber or at 6-10 minutes in both chambers. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to WT controls of the same drug treatment; (n = 2-8)



**Figure 4.** Marble burying activity of 12-month-old and 24-month-old wild type mice (WT),  $\beta 2^*$ nAChR knockout mice ( $\beta 2$ KO) and  $\beta 2^*$ nAChR heterozygous mice ( $\beta 2$ HET). Compared to 12-month-old WT mice, 12-month-old  $\beta 2$ HET mice buried a significantly more marbles. There was no effect of di-hydrobeta-erythroidine (DH $\beta$ E) treatment or interaction of genotype by age by treatment on measures of marble burying activity. Data are expressed as mean  $\pm$  SEM; \* $p < 0.05$  compared to WT controls ( $n = 3-7$  per group).

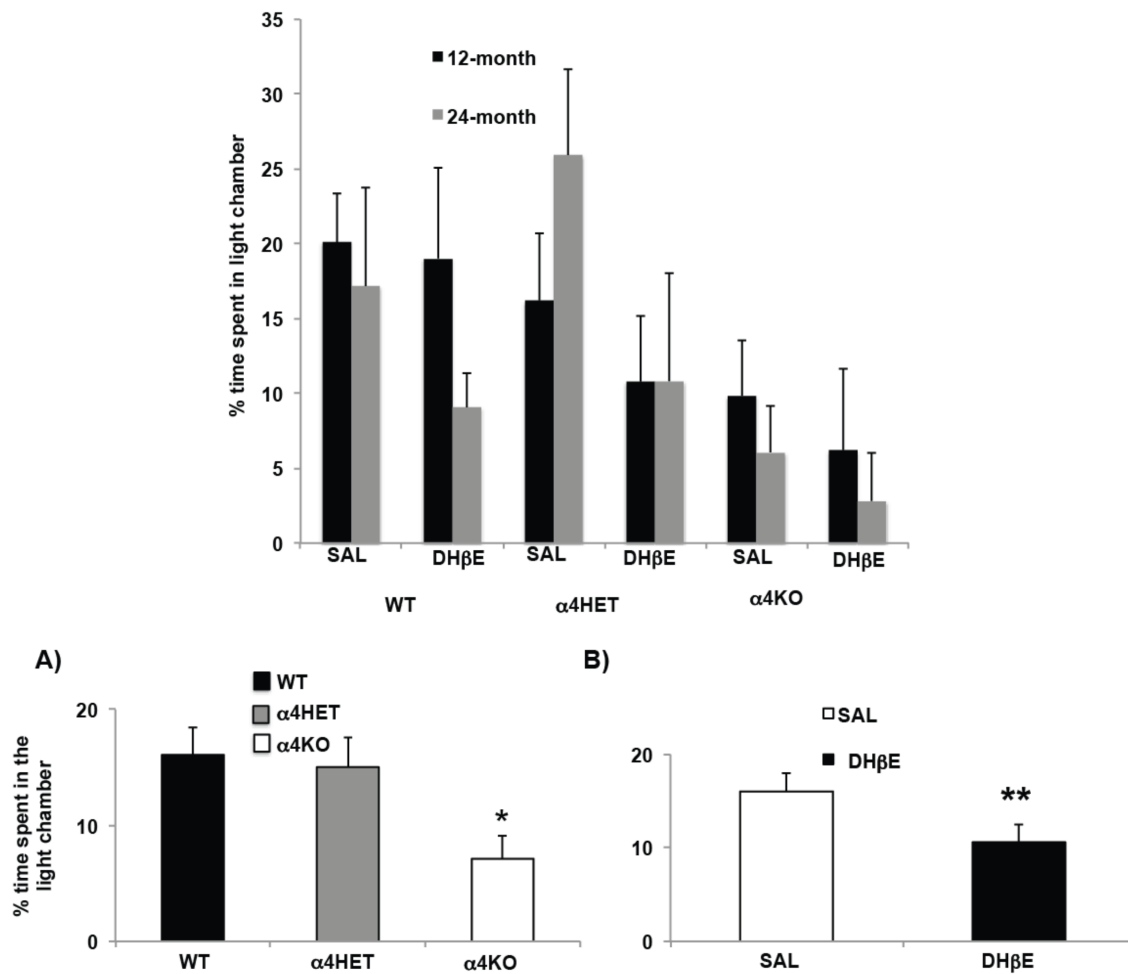


**Figure 5.** Open field activity of 12-month-old and 24-month-old wild type mice (WT),  $\beta 2^*$ nAChR knockout mice ( $\beta 2$ KO) and  $\beta 2^*$ nAChR heterozygous mice ( $\beta 2$ HET) on measures of distance traveled, time spent and rearing in the center and perimeter of the open field arena. A) Compared to WT mice,  $\beta 2$ KO but not  $\beta 2$ HET mice travelled a significantly greater distance in the perimeter of the open field. There was no effect of genotype on B) distance traveled in the center of the arena, on C, D) time spent in the perimeter or the center, or on F) rearing in the center of the open field. E) Similarly to distance traveled,  $\beta 2$ KO mice showed a significant increase in exploration of the perimeter as measured by elevated levels of rearing compared to WT mice. There was no effect of age or interaction of genotype with age on measures of open field activity. Data are expressed as mean  $\pm$  SEM; \* $p < 0.05$  compared to WT controls ( $n = 11$ -20 per group).

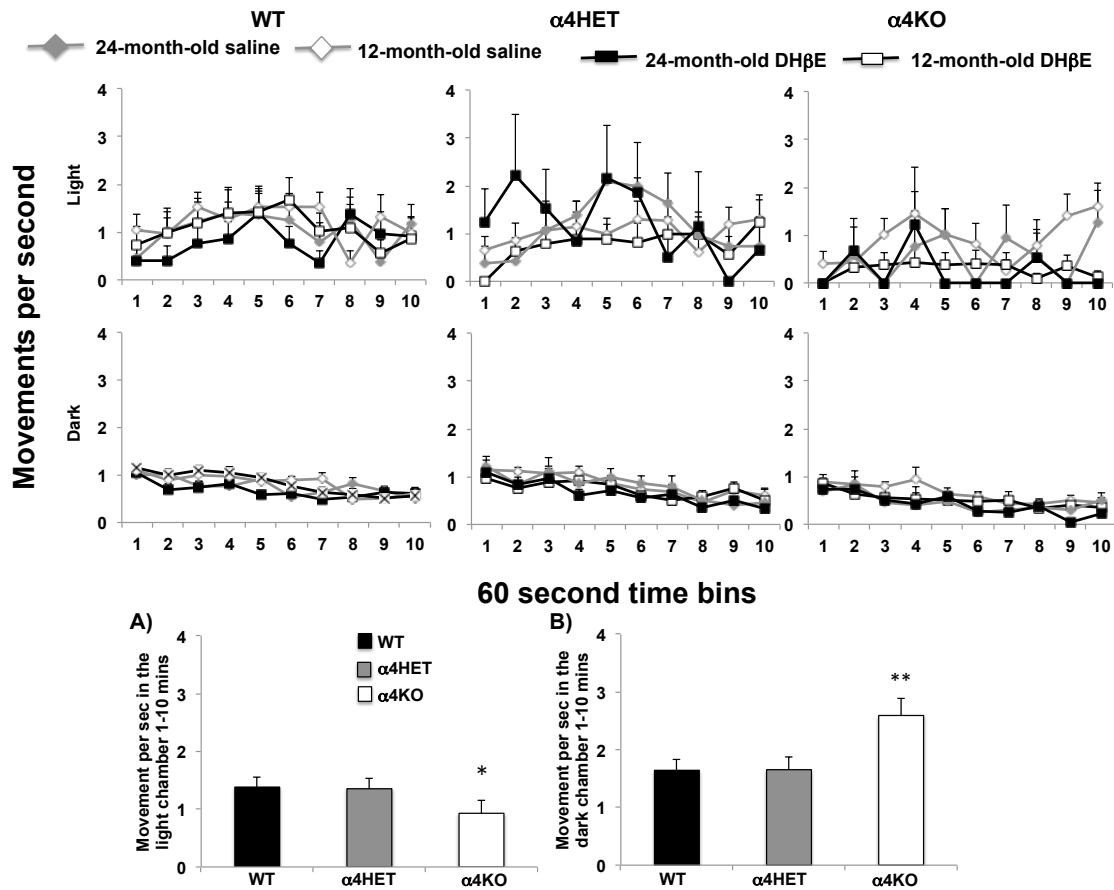


**Figure 6.** Light-dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\alpha 4^*$ nAChR knockout mice ( $\alpha 4$ KO) and  $\alpha 4^*$ nAChR heterozygous mice ( $\alpha 4$ HET) on measures of latency to enter the light chamber. A) Compared to WT mice,  $\alpha 4$ KO but not  $\alpha 4$ HET mice latency to enter the light chamber was significantly greater. Similarly treatment with di-hydrobeta-erythroidine (DH $\beta$ E) B) significantly increased the latency to enter the light chamber compared to saline (SAL) treated mice. No effect of age or interaction of age by genotype by treatment on measures of light-dark activity. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to WT; \*\* $p$  < 0.05 compared to drug controls (n = 3-10 per group).

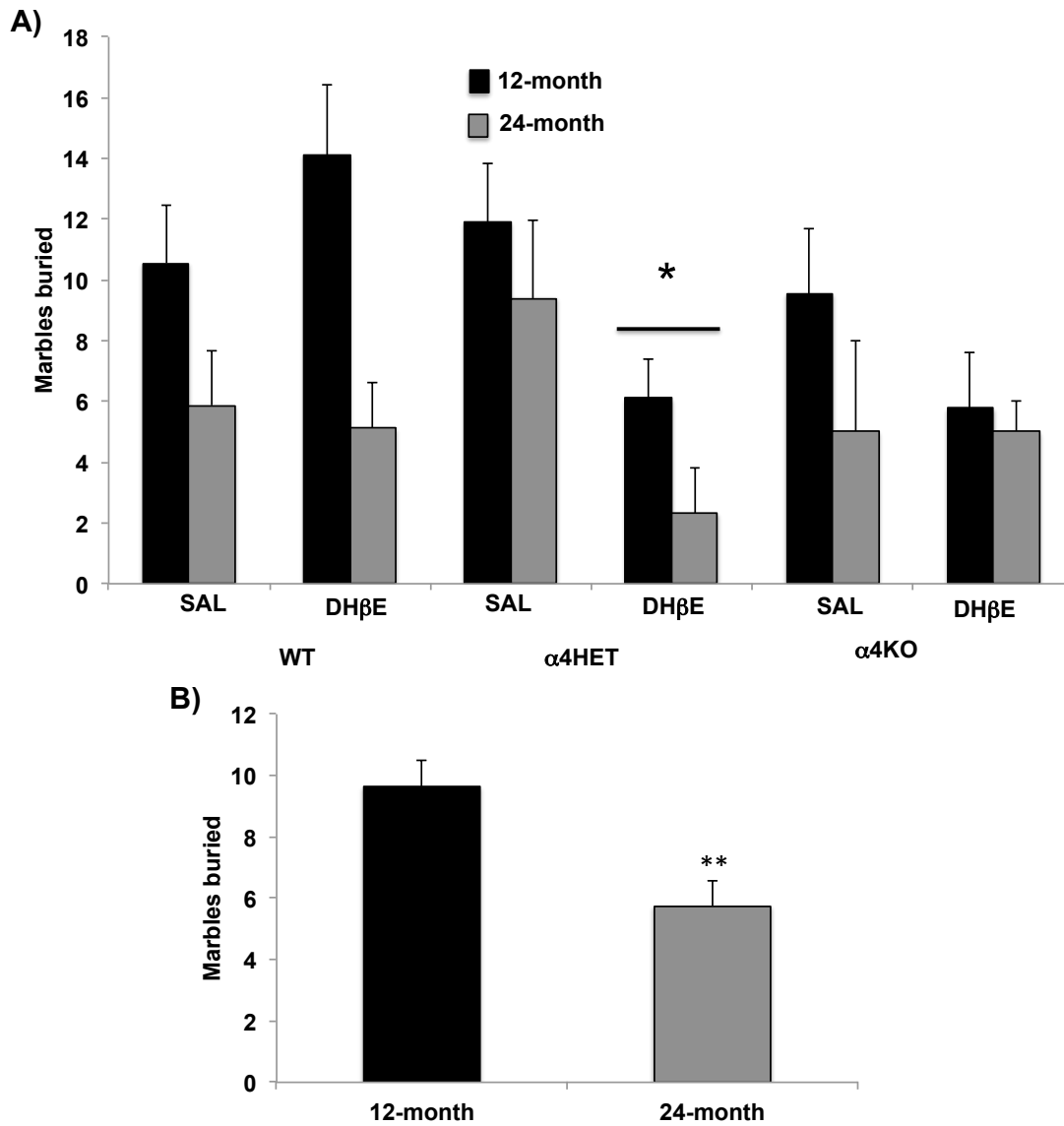




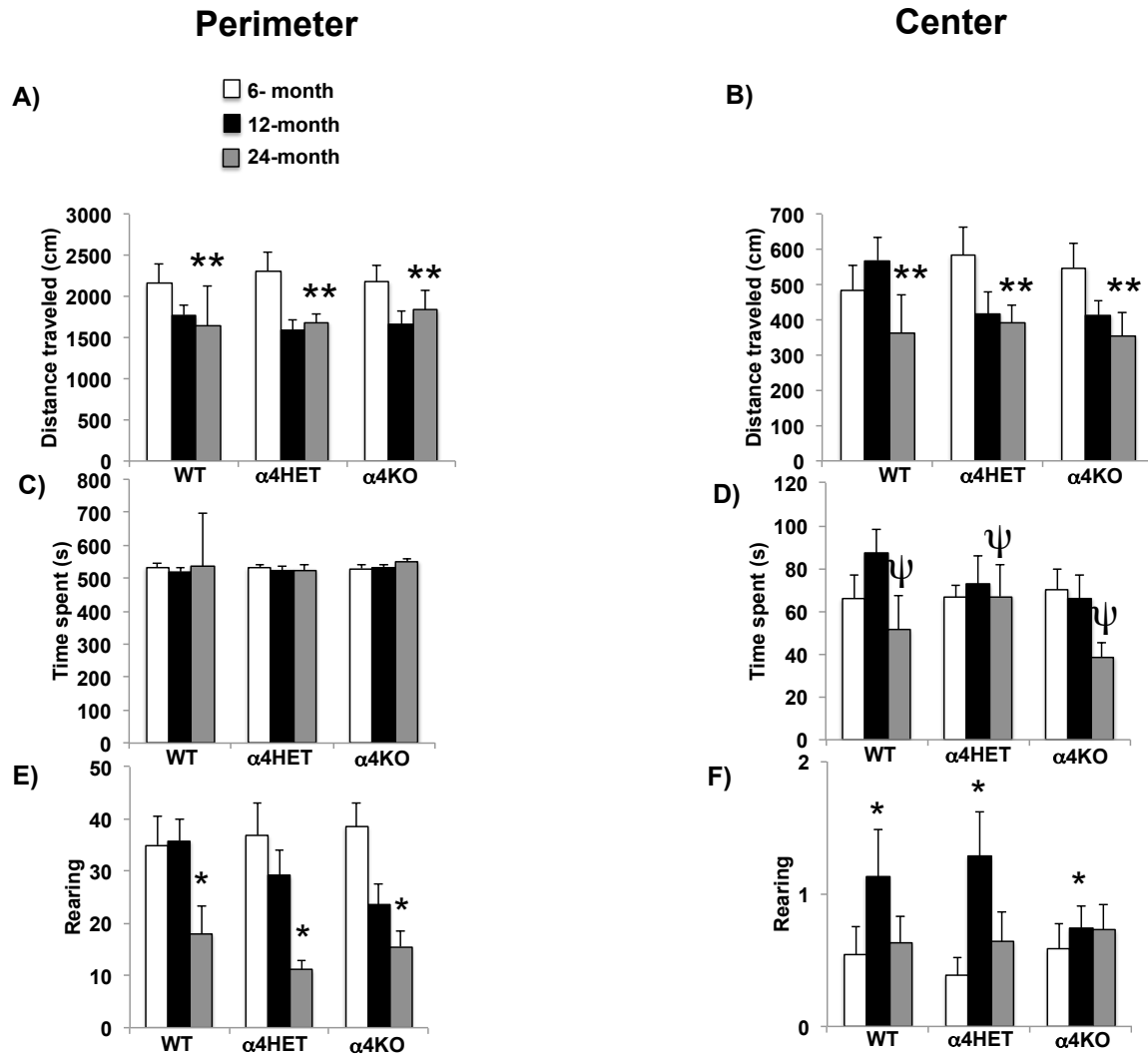
**Figure 7.** Light-dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\alpha 4^*$ nAChR knockout mice ( $\alpha 4$ KO) and  $\alpha 4^*$ nAChR heterozygous mice ( $\alpha 4$ HET) on measures of percentage of time spent in the light chamber. A) Compared to WT mice,  $\alpha 4$ KO but not  $\alpha 4$ HET mice spent a significantly decreased percentage of time in the light chamber. Similarly treatment with di-hydro-beta-erythroidine (DH $\beta$ E) B) significantly decreased the latency to enter the light chamber compared to saline (SAL) treated mice. No effect of age or interaction of age by genotype by treatment on measures of light-dark activity. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to WT; \*\* $p$  < 0.05 compared to drug controls; (n = 3-10 per group).



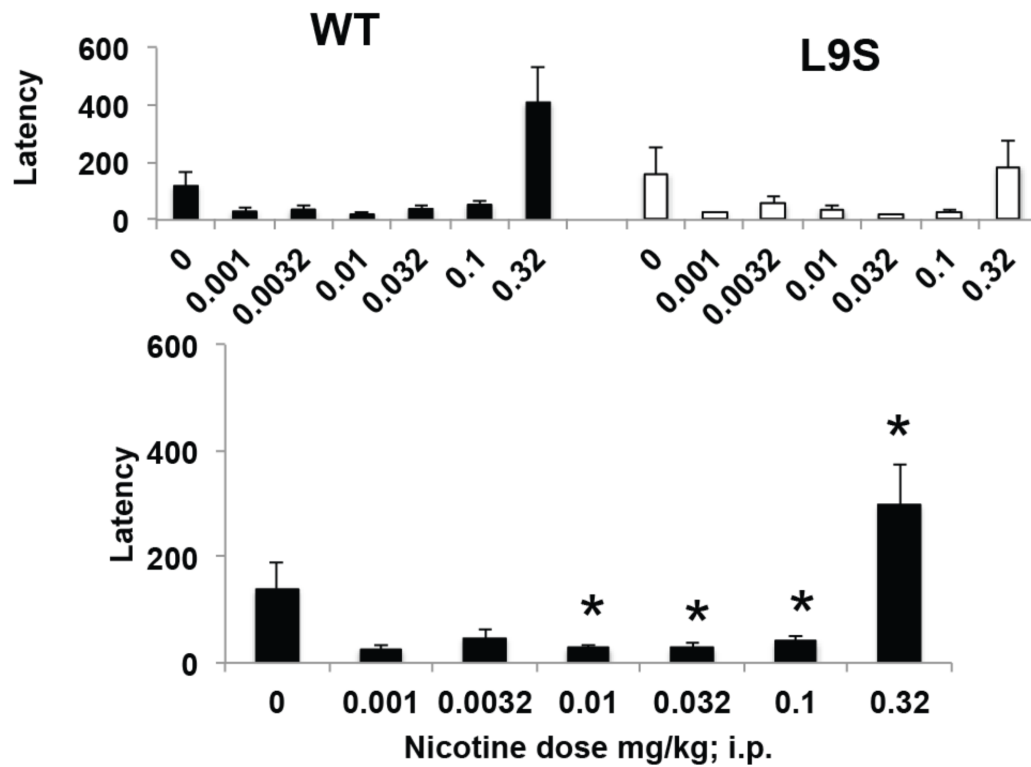
**Figure 8.** Light-dark activity of 12-month-old and 24-month-old wild type mice (WT),  $\alpha 4^*$ nAChR knockout mice ( $\alpha 4$ KO) and  $\alpha 4^*$ nAChR heterozygous mice ( $\alpha 4$ HET) on measures of movement per second in the light and dark chambers. A) Compared to WT,  $\alpha 4$ KO but not  $\alpha 4$ HET mice had decreased movement per second in the light chamber compared to WT mice and B) increased movement per second in the dark chamber compared to WT mice. Data are expressed as mean  $\pm$  SEM; \* $p < 0.05$  compared to WT controls; \*\* $p < 0.01$  compared to drug controls (n = 3-10 per group).



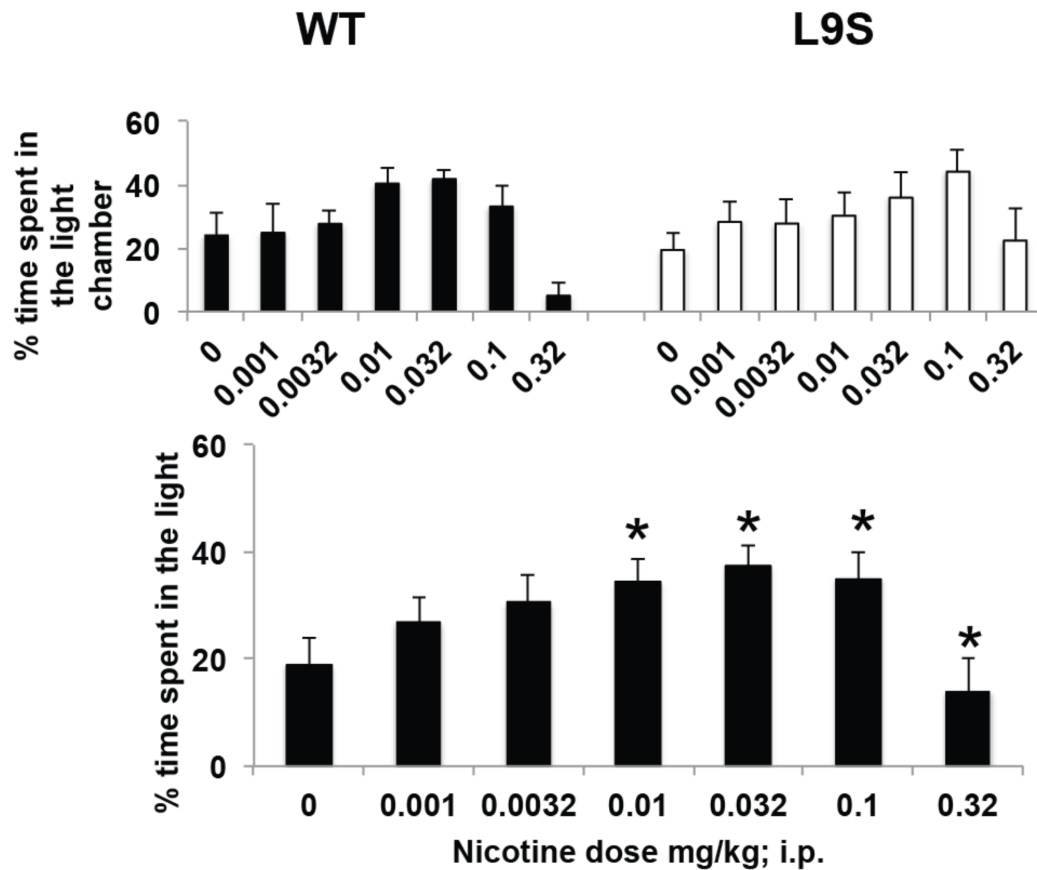
**Figure 9.** Marble burying activity of 12-month-old and 24-month-old wild type mice (WT),  $\alpha 4^*$ nAChR knockout mice ( $\alpha 4$ KO) and  $\alpha 4^*$ nAChR heterozygous mice ( $\alpha 4$ HET). A) Antagonism with di-hydrobeta-erythroidine (DH $\beta$ E) significantly reduced the number of marbles buried in  $\alpha 4$ HET mice but not  $\alpha 4$ KO compared to WT mice. B) 24-month-old mice buried significantly less marbles than 12-month-old mice. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to 12-month-old controls; \*\* $p$  < 0.01 compared to 12-month-old; (n = 2-10 per group).



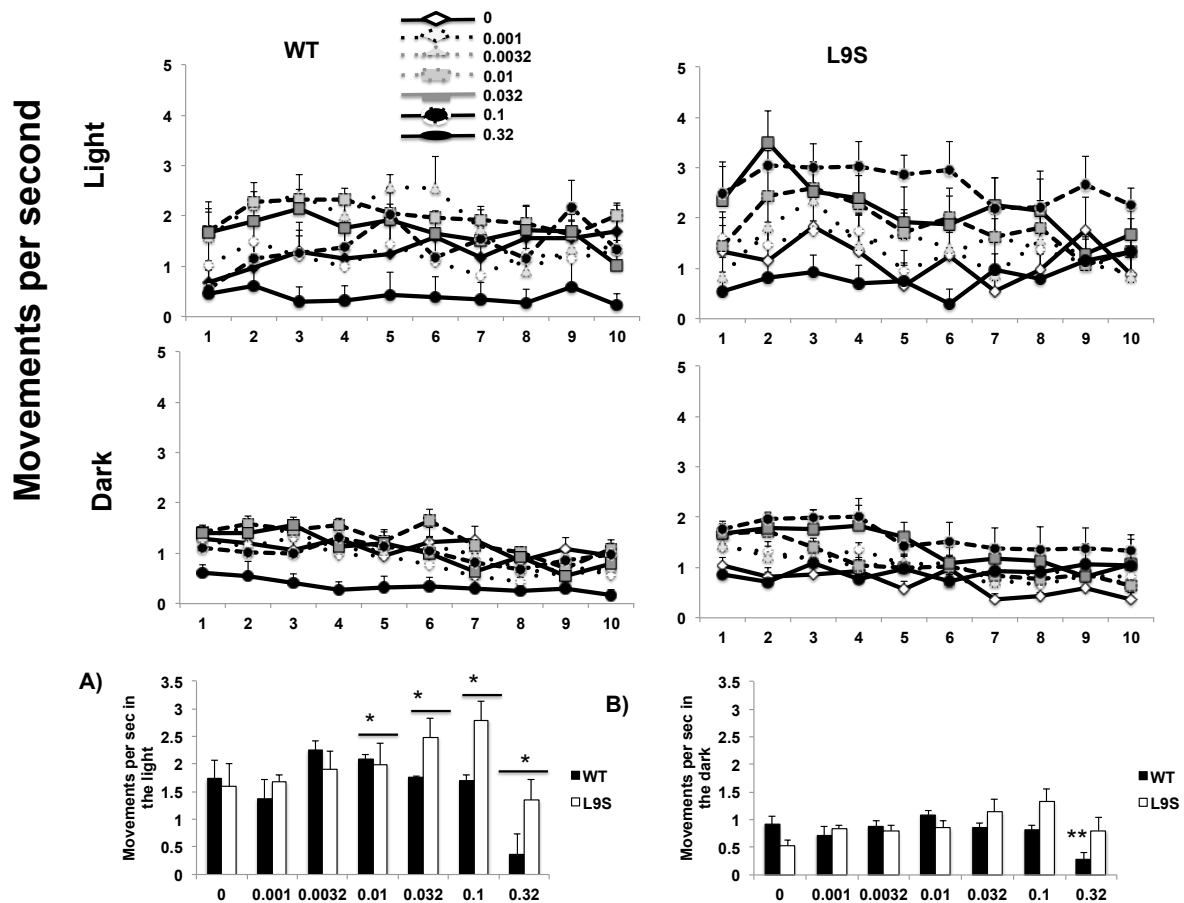
**Figure 10.** Open field activity of 6-month-old, 12-month-old and 24-month-old wild type mice (WT),  $\alpha 4^*$ nAChR knockout mice ( $\alpha 4$ KO) and  $\alpha 4^*$ nAChR heterozygous mice ( $\alpha 4$ HET) on measures of distance traveled, time spent and rearing in the center and perimeter of the open field arena. A) Compared to 6-month-old mice, 24-month-old but not 12-month-old mice travelled a significantly greater distance in the perimeter of the open field. B) Similarly 24-month-old mice and 12-month-old mice greater distance compared to 6-month-old mice in the center of the arena. There was no effect of genotype on C) time spent in the perimeter of the open field. D) A trend for 12-month-old mice to spent increased time in the center of the arena compared to 24-month old mice in the center of the open field arena. E) 24-month-old mice reared significantly longer than did 6-month-old mice in the perimeter of the open field arena F) 12-month-old mice reared significantly longer than did 6-month-old mice in the center of the open field arena. There was no effect of genotype or interaction of genotype with age on measures of open field activity. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to 6-month-old controls, \*\* $p$  < 0.01 compared to 6-month-old controls, \*\*\* $p$  < 0.001 compared to 6-month-old controls;  $\psi$   $p$  = 0.053 compared to 12-month-old; (n = 11-20 per group).



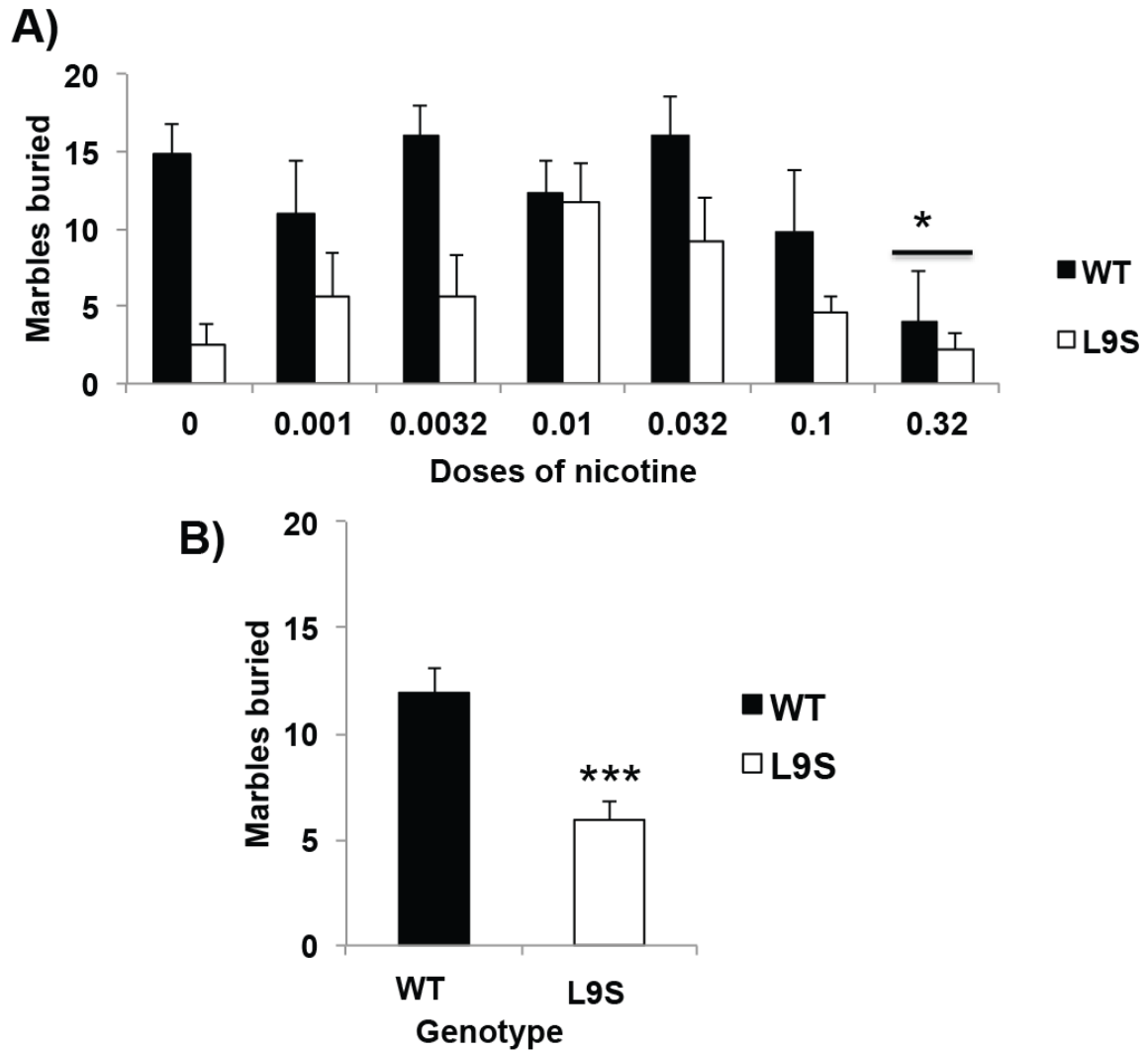
**Figure 11.** Light- dark activity of wild type mice (WT) and  $\alpha 6$ L9S nAChR mice (L9S) on measures of latency to enter the light chamber. Low doses of nicotine (0.01, 0.032 and 0.1 mg/kg, i.p.) significantly reduced latencies to enter the light chamber compared to saline. In contrast a high dose of nicotine (0.32 mg/kg; i.p.) significantly increased latency to enter the light chamber compared to saline treated mice. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to 6-month-old controls, \* $p$  < 0.05 compared to saline control; (n = 6 per group).



**Figure 12.** Light- dark activity of wild type mice (WT) and  $\alpha 6$ L9S nAChR mice (L9S) on measures of percentage of time spent in the light chamber. Low doses of nicotine (0.01, 0.032 and 0.1 mg/kg, i.p.) significantly increased time spent in the light chamber compared to saline. In contrast a high dose of nicotine (0.32 mg/kg; i.p.) significantly decreased time spent in the light chamber compared to saline treated mice. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to 6-month-old controls, \* $p$  < 0.05 compared to saline control; (n = 6 per group).

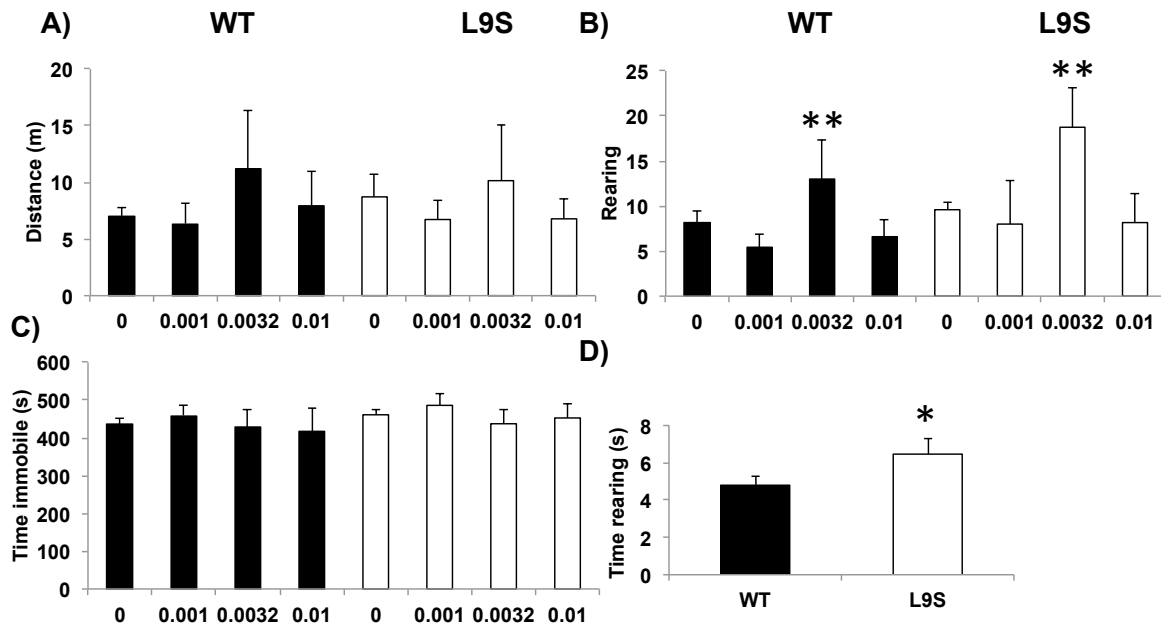


**Figure 13.** Light- dark activity of wild type mice (WT) and  $\alpha 6$ L9S nAChR mice (L9S) on measures of movement per sec in the light and dark chambers. A) Low doses of nicotine (0.01, 0.032 and 0.1 mg/kg, i.p.) significantly increased movements per sec in the light chamber compared to saline. In contrast a high dose of nicotine (0.32 mg/kg; i.p.) significantly reduced movements per sec in the light chamber compared to saline treated mice. B) WT mice treated with a high dose of nicotine showed reduced movements per sec in the dark chamber compared to saline treated WT mice. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to saline, \*\* $p$  < 0.05 compared to WT drug control; (n = 6 per group).



**Figure 14.** Marble burying activity of wild type mice (WT) and  $\alpha 6$ L9S nAChR mice (L9S) on measures of marbles buried. A) A high dose of nicotine (0.32 mg/kg; i.p.) significantly reduced the number of marbles buried compared to saline treated mice. B) L9S mice buried significantly less marbles than WT. Data are expressed as mean  $\pm$  SEM; \* $p$  < 0.05 compared to saline, \*\*\* $p$  < 0.01 compared to WT mice; (n = 6 per group).





**Figure 15.** Locomotor activity of wild type mice (WT) and  $\alpha 6$ L9S nAChR mice (L9S) on measures of distance traveled, time immobile, rearing and time spent rearing in the locomotor activity apparatus. No effect of nicotine dose on locomotor activity measures of A) distance traveled in the apparatus or C) time spent immobile. B) A low dose of nicotine (0.0032 mg/kg, i.p.) significantly increased the number or rearing behavior compared to saline treated mice. D) L9S mice spent more time rearing than did WT mice. No effect of genotype or interaction of genotype by treatment on measures of locomotor activity. Data are expressed as mean  $\pm$  SEM; \*\* $p < 0.01$  compared to saline, \* $p < 0.05$  compared to WT mice; (n = 6 per group).

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